Unraveling the journey of chromatin in lupus

- New clues for the understanding of the pathogenesis? -

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Chapter 1.

General introduction and outline of this thesis

General Introduction

Our body is involved in a daily battle with many intruding microorganisms, such as bacteria, fungi and viruses. Fortunately, we are equiped with a defense mechanism against these foreign intrudors, i.e. our immune system is trained to recognize all kind of structures, but only from invaders, not structures from the body itself. However, sometimes the immune system fails. Systemic lupus erythematosus (SLE) is the consequence of such a failure in our immune mechanism. SLE is an autoimmune disease, which means that the immune system produces antibodies against self structures, which include many different autoantigens in SLE. Since autoantibodies circulate in the blood stream, they can lead to inflammation throughout the body, such as in the skin and joints, but also in organs like the kidneys, the central nervous system, heart, lungs and blood vessels. These inflamation reactions cause the disease manifestations of SLE, which are very different between patients. Characteristic for SLE is the facial skin erythema in the form of a butterfly. The term lupus (Latin for wolf) is attributed to the thirteenth century physician Rogerius who used it to describe erosive facial lesions that were reminiscent of a wolf's bite. In the United States 1.4 million people suffer from SLE, but the prevalence of SLE in the world is not exactly known, however the number of SLE patients seemed to be increased during the last decades. Most likely several factors play a role in the development of the disease, such as environmental (sun light), hormonal, genetic factors and infections. SLE is more commonly found in woman (90%) and can develop at each age, but most frequently between 20-40 years. Since the cause is not known yet, the disease itself cannot be cured. Presently, corticosteroids are the primary therapy for the majority of patients with SLE. Antimalarials are used principally for patients with skin and joint involvement and cytotoxic immunosuppressive drugs are used for patients with glomerulonephritis, systemic vasculitis, and other severe life-threatening manifestations.

History of SLE research

Despite decades of research, the cause(s) of SLE still remain a jig-saw puzzle. The history of modern research on SLE began with the discovery of the LE cell by Hargraves and colleagues in 1948 (3). They postulated that: "this cell is the result of phagocytosis of free nuclear material with a resulting round vacuole containing this partially digested and lysed nuclear material". In the 1950s, Friou applied the technique of indirect immunofluorescence to demonstrate the presence of antinuclear antibodies in the blood of patients with SLE (4). Subsequently, autoantibodies against deoxyribonucleic acid (DNA), extractable nuclear antigens

(ENA), i.e. nuclear ribonucleoprotein (nRNP), Sm, Ro, La, and anticardiolipin were described. A major advance in SLE research was the development of animal models for SLE. The first animal model for SLE was the F1 hybrid of New Zealand Black and New Zealand White mouse (NZWBF1) (5). These mice developed symptoms similar to SLE patients, including the formation of anti-nuclear autoantibodies and the development of lupus nephritis. Later SLE disease was also found in certain strains containing a gld or lpr mutation (6), which results in a deficiency for the Fas ligand (FasL) or the Fas protein, respectively. An abnormal Fas-FasL pathway, which is involved in the apoptosis and removal of certain cells of the immune system, leads to the persistence of both autoreactive T- and B-cells. Importantly,

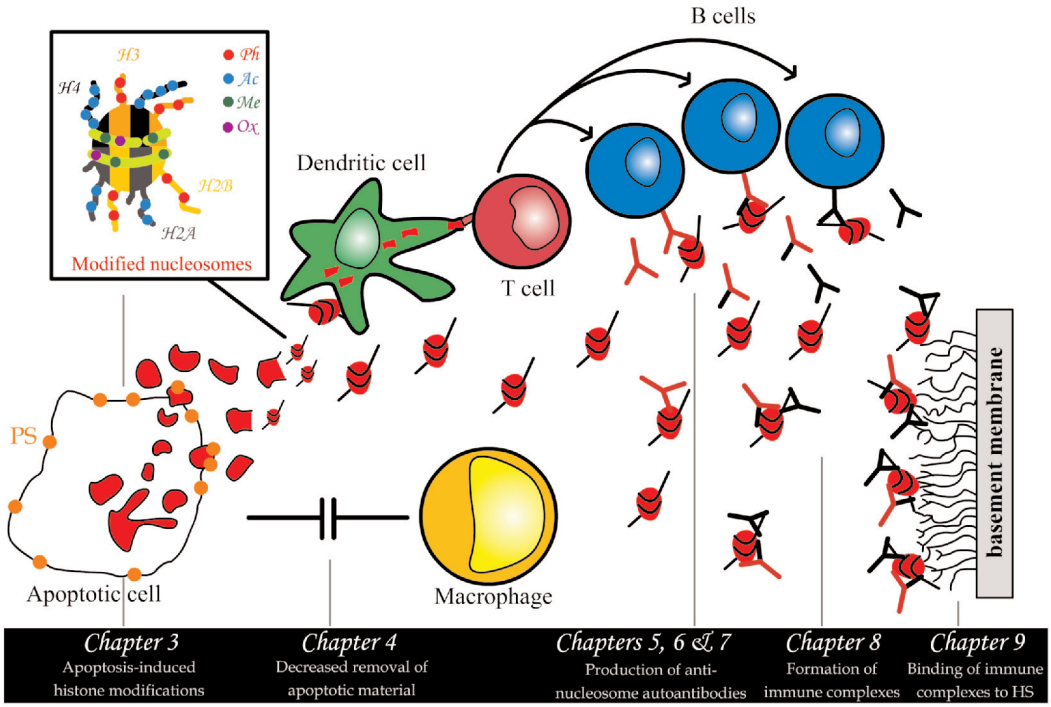

Figure 1. The role of nucleosomes in the development of the anti-chromatin response and glomerulonephritis in lupus: A disturbed apoptosis or removal of apoptotic cells leads to the release of modified apoptotic material, including nucleosomes. Modifications on the nucleosome associated with apoptosis are depicted in the inset. Dendritic cells digest the nucleosomes and present (modified) histone peptides to histone-specific T cells. Activated histone-specific T cells, in cooperation with activated DNA- and nucleosome-specific T cells, stimulate B-cells to produce anti-histone, nucleosome-specific and anti-DNA autoantibodies. Finally, anti-nucleosome/nucleosome complexes are formed, which bind to negatively charged residues in heparan sulfate, present in basement membranes, such as the glomerular basement membrane in the kidney.

PS: phosphatidyl serine, Ph: phosphorylation, Ac: acetylation, Me: methylation, Ox: oxidation

onset and severity of symptoms in mice bearing the lpr or gld mutation is dependent on the genetic background (7). These and other SLE mice models have led to a much better understanding of the processes that lead to the development of the disease. SLE was found to be driven by autoantigens and was dependent on T and B cells. Until now, more than 100 autoantigens have been identified that are targeted by SLE autoantibodies (8). The autoantigens that are targeted by autoantibodies are different between patients. Since anti-DNA autoantibodies were found in nearly all patients, these autoantibodies were considered as the serological marker for SLE. However, DNA is not immunogenic by itself, only when bound to proteins (i.e. histones or viral proteins) it can induce an anti-nucleosome and anti-DNA response (9, 10). Therefore, the DNA/histone complex, the nucleosome, was proposed as the major autoantigen (11). Indeed, the last decade cumulating evidence points to the nucleosome as the major autoantigen that drives the autoimmune response in SLE (12, 13), as depicted in Figure 1.

The nucleosome

The nucleosome is the basic unit of chromatin, the nuclear structure in which 2 meters of DNA is densily packed (Figure 2a). Each nucleosome is made up of 146 base pairs (bp) of double stranded (ds)DNA wrapped twice around a histone octamer formed by pairs of the histones H2A, H2B, H3 and H4 (Figure 2b). In chromatin, nucleosomes are connected by 15-80 base pairs of linker DNA, to which histone H1 is attached. The nucleosome contains domains that are positively charged due to basic amino acids especially in the N-terminal tails of the core histones, and domains that are negatively charged due to the negative charge of DNA. These charges enable nucleosomes to bind easily to other positively or negatively charged molecules. Especially, the histone tails protruding from the nucleosome core particle may act as ‘sticky fingers’ for negatively charged molecules, such as heparan sulfate.

Normally, i.e. in their physiological context, histones can be heavily modified especially at the N-terminal tails. These histone modifications include phosphorylation of serine or threonine, acetylation of lysine or arginine, (mono-, di- or tri-) methylation of lysine or arginine, citrullination of arginine, ubiquitination of lysine, transglutamination of glutamine or lysine and sumoylation of sofar unidentified residues (Figure 2c). It has now become clear that a histone modification or a combination of histone modifications in a certain chromatin region greatly determines the activity of that domain, i.e. recruitment of other proteins, involved in transcriptional silencing, activation, DNA repair, mitosis or meiosis. Furthermore, one histone modification may effect the presence or absence of other

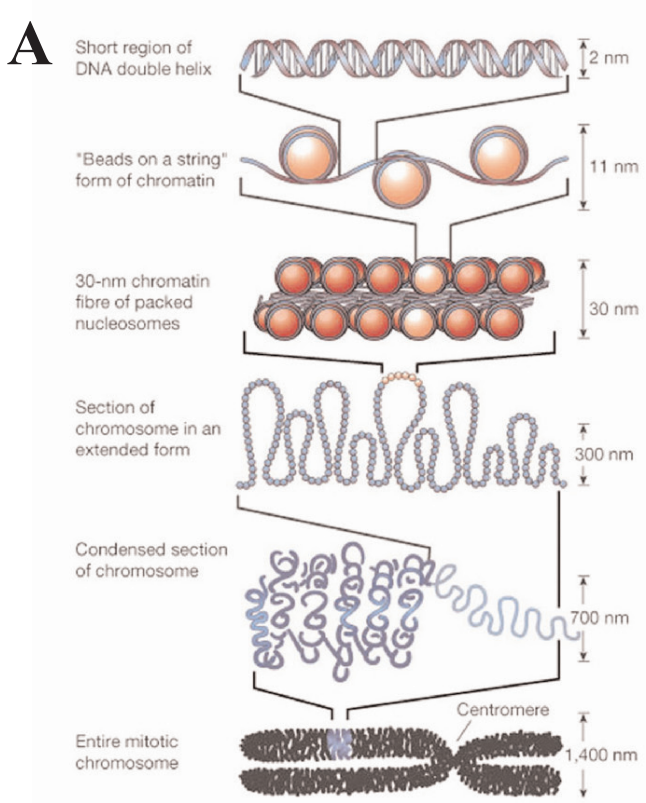

Figure 2. Chromatin structure and histone modifications. **A.** From the the 'double helix' to the chromosome structure, reproduced from Richmond et al. in Nature (2003) (1). **B.** 3D model of the nucleosome (PDB: 3AOI) (1). Protruding tails of the histones are mostly not present in this model. However, a part of one tail of histone H3 is present, where the SAPAT epitope (Chapter 7) is located (marked by an arterisk). C. Modifications on the core histones as described in the literature. Specific modifications linked to apoptosis are underlined.

Abreviations used are:

Ac (acetylation)
P (phosphorylation)
Me (methylation)
Ub (ubiquitination)
Tg (transglutamination)

histone modifications. These observations led to the proposal of a histone code (14), which links specific histone marks to distinct (cell) biological processes.

Apoptosis-induced autoantigen modifications

Apoptosis is a mechanism of programmed cell death that leads to the removal of cells from our body without exposing their dying contents to the our system, including the immune system. During apoptosis, many proteins are cleaved by proteases, in addition DNA and RNA are cleaved by endonucleases. These proteases involve a large family of so called caspases that are able to cleave a still increasing list of about 300 proteins with various functions (15). The caspase substrates include caspase family members that become activated upon proteolytic cleavage thereby activating the downstream caspase cascade pathway of cell death. Inactive caspase activated DNase (ICAD) also becomes activated to caspase activated DNase (CAD) by proteolytic cleavage by caspases. This leads to the cleavage of chromatin into individual nucleosomes. Cytotoxic T cells or natural killer cells can induce apoptosis via another pathway by the release of protease granzyme B, cleaving a number of proteins that are essential for cells to survive. In fact susceptibility for cleavage by granzyme B greatly determines whether a non-histone protein can become an autoantigen (16, 17). Although it has been demonstrated that chromatin-associated SLE autoantigens are substrates for caspases and/or granzyme B, not much is known about proteolytic processing of the nucleosome and/or the separate core histone proteins by caspases and/or granzyme B.

As already mentioned, histones can be heavily modified, which also includes modifications induced during apoptosis. Recently, a caspase-actived kinase (twenty kinase) has been shown to phosphorylate histone H2B at serine 14 (S14) during apoptosis using several inducers for apoptosis in various cell lines(18). In addition, apoptosis-induced phosphorylation has been shown for histone H2B at other serine residues (S32 and S35), for histone H2A at unknown residues, for the minor histone H2A variant, H2A.X, at S139, and for histone H3 at S10 or S28 (18-24). Both apoptosis-induced histone deacetylation and acetylation have been described (25-28). For instance, cis-platin induced apoptosis resulted in global hyperacetylation of histones. Moreover, treatment by several histone deacetylase (HDAC) inhibitors, which also results in hyperacetylation of histones, induced apoptosis, while HDAC inhibitors and apoptosis inducers can act synergistically. Other specific apoptosis-induced modifications that have been described include poly(ADP-)ribosylation and dephosphorylation of histone H1, de-ubiquitination of histone H2A and transglutamination of H2B (29-32). Recently, the involvement of histone H1 variant

H1.2 in the induction of apoptosis has also been reported (33). Until now, a clear histone code linked to the process of apoptosis has not been identified. Besides histones, DNA can be modified by cleavage, methylation or oxidation by radicals (34, 35). Despite the identification of apoptosis-induced histone/DNA modifications, there is little knowledge about the relevance of these modifications for the recognition by SLE autoantibodies.

Removal of apoptotic cells

A paramount report by Cascialo-Rosen and co-workers showed that autoantigens become clustered in apoptotic blebs after exposure of keratinocytes to UV light (36). The larger blebs contained DNA, SS-A (Ro), SS-B (La) and the small nuclear ribonucleoproteins, while the smaller blebs contained fragmented endoplasmatic reticulum (ER), ribosomes and SS-A (Ro). This observation provided for the first time a mechanism how seemingly unrelated and deeply hidden nuclear and cytoplasmatic antigens can serve as autoantigens that challenge the immune system. In addition, a recent report shows that anti-nucleosome autoantibodies can bind to nucleosomes exposed at the surface of apoptotic blebs (37). The expression of SLE-targeted autoantigens at the surface of apoptotic cells may be an important intitial event in the induction of autoimmunity. Normally, apoptotic cells are swiftly removed by either professional (i.e. macrophages) or non-professional phagocytes (i.e. neighbouring cells) before the formation of apoptotic blebs. However, apoptosis at the wrong environment and/or at the wrong time-point can lead to an overload of apoptotic cells for the local cleaning machinery, resulting in the release of histones and nucleosomes with apoptosis-induced modifications (11, 13, 38, 39).

Presentation of phosphatidyl serine on the cell surface, is the first ‘eat me’ signal for apoptotic cells. However, recognition of apoptotic cells is a complex process, involving many phagocytic receptors, bridging molecules and markers on the apoptotic cell (40). Studies involving these molecules have shown that a disturbed removal of apoptotic cells results in the development of SLE (39, 41). Mice deficient for molecules involved in apoptotic cell removal, developed anti-nuclear autoantibodies, glomerular deposits containing immunoglobulins and complement factors, and in some cases glomerulonephritis. In addition to the direct removal of apoptotic cells, a ‘back-up’ system seems to exist in case this removal is impaired. Here, apoptotic material and potential autoantigens such as nucleosomes are either masked and tethered for removal by C1q, serum amyloid P (SAP) or pentraxin 3 (PTX3), or directly broken down by DNaseI. Mice deficient for these molecules also developed a SLE-like syndrome. In SLE mouse models, like the

MRL/lpr and (NZBxNZW)F1, there is evidence for an impaired removal of apoptotic cells (42, 43). In SLE patients it has been demonstrated that the phagocytosis of apoptotic cells is disturbed (44-46). In addition, increased levels of apoptotic cells have been found in plasmas from SLE patients, however this was not correlated to disease activity and was also found in non-SLE patients (45, 47, 48). Nucleosomal material (i.e. DNA and histones) has also been detected in plasmas of SLE patients (49, 50) and SLE mice (51).

Immunogenicity of apoptotic cells and nucleosomes

Nucleosomes, present due to an impaired removal of apoptotic cells, are able to inhibit the uptake of apoptotic cells (52), which could lead to an perpetuing circle. Moreover, the release of apoptotic and thus modified nucleosomes may lead to the activation of the immune system. Macrophages act as the professional garbish men, without inciting an inflammatory response. The uptake of apoptotic cells by macrophages suppresses the release of pro-inflammatory cytokines and stimulates the production of anti-inflammatory cytokines (40). Ineffective removal of apoptotic cells leads to the persistence of apoptotic material in the circulation, which can be encountered by dentritic cells (DCs), thus promoting a pro-inflammatory response. DCs are the professional antigen presenting cells and mature in response to 'danger signals'. Immature DCs are capable of ingesting large amounts of material from apoptotic cells, subsequently, upon maturation DCs will present the processed apoptotic material to T cells. Apoptosis-induced modifications could enhance the immunogenicity of nucleosomes. Indeed, DCs have been shown to present apoptotic material to T cells (53). In case of nucleosomes, DNA is only immunogenic when it is attached to proteins, like histones or viral proteins, leading to the uptake and presentation of peptides from these proteins. Indeed, histone-specific and nucleosome-specific T helper cells have been identified (54), which also resulted in the identification of several T cell epitopes on the core histones (55-58). In contrast to the normal immune response, T cell and B cell epitopes frequently overlap in SLE (59, 60). The nucleosomal and/or histone T cell epitopes that have been identified in humans and mice are located in histone H2A (amino acids (aa) 34-48), H2B (aa 10-33), H3 (aa 85-114) and H4 (aa 14-39, aa 49-63 and aa 71-99). Another T cell epitope has been found in H3 (aa 53-85) for mice that were immunized with apoptotic cells. Some established histone modifications are located in the identified T cell epitopes, i.e. acetylation of K12, K15 and K20 and phosphorylation of S14 in H2B, methylation of K79 in H3, and acetylation of K16 and methylation of K20 in H4. However, only the phosphorylation of S14 in H2B has been related to apoptosis so far (18). The lack of modifications on the histone peptides that have been used

for the identification procedure of T cell epitopes may have hampered the identification of modified T cell epitopes in these potentially heavily modified regions. Histone-specific T cells are able to provide help for the production of nucleosome-specific and anti-DNA autoantibodies by stimulating T cells specific for nucleosomes and DNA (54, 60), a process known as 'antigen spreading'. The majority (50%) of these T cells have been identified as nucleosome-specific, again underlining the importancy of the nucleosome as autoantigen (60). Antigen spreading can also explain why activation by apoptosis-modified chromatin may result in the development of autoantibodies specific for 'normal' chromatin. Histone-, DNA-, and nucleosome-specific T cells augment the production of anti-DNA, anti-histone and nucleosome-specific autoantibodies by B cells. A defective apoptosis can also play a role in the survival of autoreactive T and B cells (39), as has been shown in mice with mutations in genes encoding molecules involved in the regulation of T and B cell apoptosis. Mice deficient for Fas, Fas ligand, Bim or TACI, or mice overexpressing Bcl-2 or Blys developed a SLE-like disease. Seemingly contradictive to the earlier suggested increased rate of apoptosis leading to the release of apoptotic material, these mice do not sufficiently remove their autoreactive T and B cells. Importanly, both mutations in genes encoding proteins involved in the removal of apoptotic cells and genes encoding proteins involved in the apoptosis of (autoreactive) cells leads only to the development of autoimmune disease in certain genetic backgrounds. This latter fact underlines the importance that SLE is a multifactorial disease.

Nucleosomes and anti-nucleosome autoantibodies as complex troublemakers in the development of SLE

As mentioned, the release of modified nucleosomes leads eventually to the production of anti-nucleosome autoantibodies. These autoantibodies can be divided into three categories: i) antibodies which recognize DNA (anti-DNA); ii) antibodies which recognize histones (anti-histone); and iii) antibodies which exclusively or predominantly recognize nucleosomes or subnucleosomal structures (nucleosome-specific). Several studies regarding anti-nucleosome autoantibodies have been performed in both SLE mice and patients. In total 1356 SLE patients and 1807 patients with other rheumatic diseases have been tested (summarized in Table I). Anti-nucleosome autoantibodies are found in the majority of SLE patients, with an average prevalence of 63%. Levels of anti-nucleosome (and anti-DNA) autoantibodies correlate with disease activity and the presence of lupus nephritis (72, 79). Especially, anti-nucleosome autoantibodies have been demonstrated to be specific for SLE (68, 70), although ome reports described that these autoantibodies

Table I. Anti-nucleosome autoantibodies in SLE patients and lupus mice

Reference	Analyzed population	Prevalence	Specificity[a]	Correlation with kidney disease[b]	Technique
Tan et al.(61)	79 patients	73%	96%	ND[h]	LE cell
Robitaille et al. (62)	36 patients	58%	95%	Yes	Immuno-ppt
Fritzler et al. (63)	20 patients	60%	ND	ND	Reconstitution
Burlingame et al. (64)	40 patients	78%	ND	Yes	ELISA
Chabre et al. (65)	40 patients	48%	ND	No[c]	ELISA
Karsh et al. (66)	71 patients	86%	86%	ND	ELISA
Suer et al. (67)	295 patients	58%	99%	ND	ELISA
Amoura et al. (68)	120 patients	72%	90%	Yes[d]	ELISA
Hmida et al. (69)	32 patients	81%	98%	ND	ELISA
Bruns et al. (70)	136 patients	56%	97%	Yes[e]	ELISA
Min et al. (71)	129 patients	76%	ND	Yes	ELISA
Cervera et al. (72)	100 patients	69%	92%	Yes	ELISA
Benucci et al. (73)	48 patients	38%	ND	Yes[c]	ELISA
Kiss et al. (74)	109 patients	39%	ND	Yes	ELISA
Horak et al. (75)	52 patients	60%	ND	No[c]	ELISA
Ravirajan et al. (76)	33 patients	73%	ND	No[c]	ELISA
Schett et al. (77)	16 patients	45%	95%	No[c]	ELISA
Fisher et al. (78)	>500 MRL/lpr mice[f]	100%	ND	ND	ELISA
	MRL/+ mice[g]	72%			
	NZBWF1 mice[g]	61%			
	BxSB mice[g]	69%			

[a] Only the controls with connective tissue disease or infectious disease were counted, groups of normal samples were not included, the average specificity in the normal group was nearly 100%
[b] In some cases a correlation was mentioned but no statistical test was performed
[c] A correlation with disease activity was found
[d] IgG3 exclusively present in SLE and related to lupus nephritis and disease activity
[e] Correlated with diseasease activity and psychosis
[f] Increasing titer with age, 50% IgG2a, 30% IgG2b, 10% IgG1 and 10% IgG3
[g] Subclass distribution similar and titers lower than in MRL/lpr mice
[h] ND: not determined

were also present in patients with scleroderma or mixed connective tissue disease (68). However, this latter observation may be related to the contamination of for instance topoisomerase I in the applied nucleosome preparations (67). Anti-histone autoantibodies are more commonly found in drug-induced lupus (DIL), but they are neither as sensitive nor specific for SLE and DIL as anti-nucleosome autoantibodies (80). The methods commonly applied to detect anti-DNA autoantibodies are an immunoprecipitation assay of radioactive-labelled DNA, called the Farr assay, or on immunofluorescence staining test using *Crithidia lucilae*, which has no histones, as substrate. Recently, anti-DNA and especially anti-nucleosome, ELISAs have become commonly available and accepted for diagnostic testing, but it should be mentioned that these tests also measure low avidity antibodies (81). Although considerable progress has been made in recent years, the anti-nucleosome response has been poorly characterized for several reasons. Studies show that a number of the patients posess anti-nucleosome reactivity, without anti-DNA or anti-histone

reactivity (65). Indeed, after absorption of sera by collumns with covently coupled DNA and histones, 25-60% of the anti-nucleosome reactivity was due to anti-DNA reactivity and 10-20% to anti-histone reactivity. In this case, 20-60% of the anti-nucleosome reactivity was due to really nucleosome-specific autoantibodies. These nucleosome-specific autoantibodies have been implicated in the development of lupus nephritis (82) and seem to appear early in disease before anti-DNA autoantibodies arise (83, 84). However, anti-DNA and anti-histone autoantibodies will also bind when nucleosomes are used as antigen for the analysis of patients sera. Moreover, anti-nucleosome/nucleosome complexes can bind to DNA (via histones) and histones (via DNA), so aborption by DNA and histone columns could also lead to the removal of nucleosome-specific autoantibodies. Because of these limitations, a method bypassing the absorption would be of great importance. The use of synthetic overlapping peptides has revealed epitopes for anti-histone and anti-spliceosome autoantibodies. For obvious reasons, this method cannot be used for nucleosome-specific autoantibodies, since these antibodies recognize conformational epitopes partially consisting of DNA and of histone proteins. For such epitopes, mimotopes should be identified. Mimotopes are peptides that mimick partially or completely non-protein structures, such as carbohydrates or DNA. The selection of mimotopes for nucleosome-specific autoantibodies can be performed by screening random peptide phage display libraries with these autoantibodies (Fig 3a). These libraries contain a random peptide fused to one of the phage coat proteins, with a complexity of about 10^8-10^{12} random sequences (Figure 3b). Random peptide phage display has been succesfully applied to identify mimotopes for antibodies against carbohydrate epitopes, which have a great potential for the development of vaccins (85, 86). Recently, this technique has also been aplied in several studies to identify mimotopes for SLE-derived anti-DNA topes, containing negatively charged, positively charged and/or aromatic residues. Further autoantibodies (87-89) (Table II). This led to the identification of completely different mimostudies with the D/E-W-D/E-Y-S/G mimotope has revealed the finding that a peptide comprising this mimotope sequence could prevent the binding of anti-DNA autoantibodies to the GBM (87). This peptide could also induce SLE in BALB/c and (NZBxNZW)F1 mice (90, 91). In addition, cross-reactivity of anti-DNA autoantibodies with an D/E-W-D/E-Y-S/G epitope in the NR2 glutamate receptor in the brain was reported (92). Mimotope peptides for nucleosome-specific autoantibodies may be used for diagnostic applications to monitor nucleosome-specific autoantibodies, or to evaluate their biological effect in normal and SLE mice. For instance, these mimotopes could be used to induce tolerance or to prevent the formation of anti-nucleosome/nucleosome complexes by competing with the nucleosome for the binding to the nucleosome-specific antibody.

The formation of anti-nucleosome/nucleosome immune complexes forms

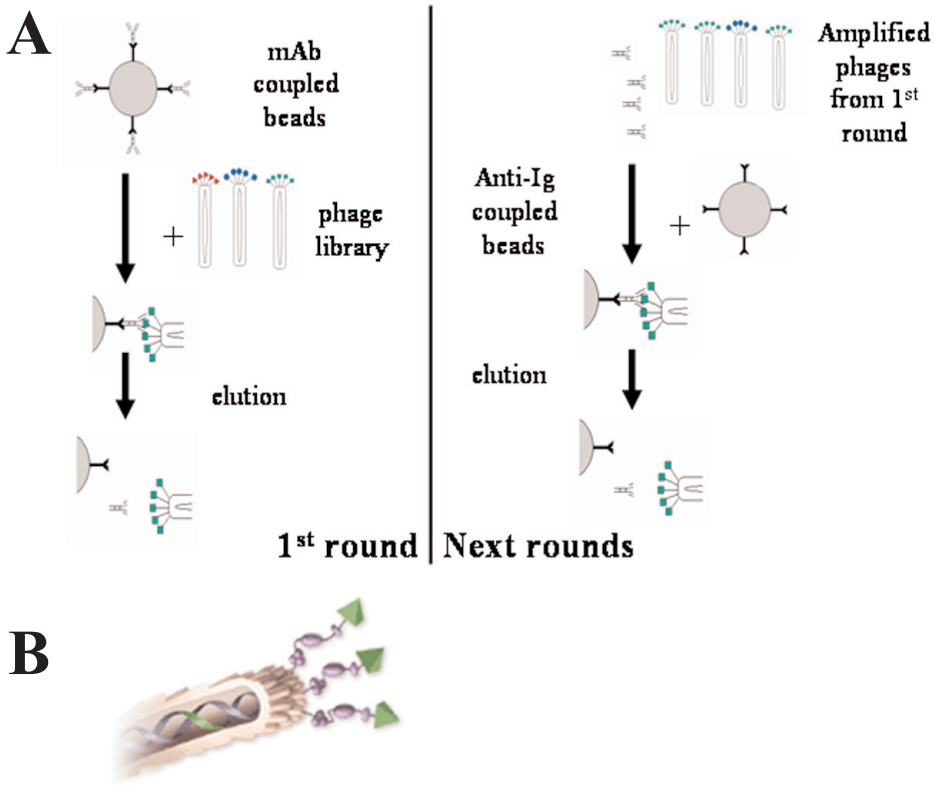

Figure 3. Random peptide phage display. **A.** Screening of random peptide libraries. In the first round, a portion of the phage library is added to a monoclonal antibody (mAb) coupled to magnetic beads. After washing, bound phages are eluted and amplified. In the next rounds, phages are first incubated with a decreasing concentration of uncoupled mAb and phage/mAb complexes are captured by Ig-coated mangetic beads, to remove phages selected for the Ig-coupled beads. Individual phage clones are picked and sequenced, and one or more motifs are deduced from these sequences. **B.** Phage containing a DNA insert (green) encoding a random protein sequence fused to the pIII coat protein of the phage (green triangles) (2).

Table II. Mimotopes for anti-DNA autoantibodies

Sequence	Library	Antibodies	Ref
D/E W D/E Y S/G	10-mer fused to pIII	R4A	(87)
R L T S S L R Y N P	15-mer fused to pIII	Polyclonal anti-DNA from patients	(89)
D C T x N T (Hy) (Hy) C Q L/T/D x E	16-mer constrained fused to pVIII	F14.6	(88)
(Hy) T G D C W/G Y/G G W G/Q x x C		J20.8	
D C x x x x W Q C		J20.8	

(Hy), green = hydrophobic residue
Red = positively charged residue
Blue = negatively charged residue
Cystein residues are underlined

the final phase for the role of nucleosomes in the development of SLE. As mentioned already, nucleosomes contain both negatively (i.e. DNA) and positively (i.e. histones) charged regions. Binding of anti-DNA or nucleosome-specific antibodies to the negatively charged regions in the nucleosome, could lead to masking of the negative charge and an enhanced binding of the formed complexes to negatively charged molecules. Previous research has shown that anti-nucleosome/nucleosome complexes can bind via the positive charges in the histones to heparan sulfate, which is present in the glomerular basement membrane (GBM) and contains many negative charged residues (93-95). The binding of complexes to the GBM initiates the development of local inflamation in the glomerulus, resulting in lupus nephritis, the most serious manifestation of SLE.

Outline of this thesis

The major aim of this thesis was to identify and characterize the epitopes for nucleosome and histone-specific autoantibodies and to evaluate their role in the lupus autoimmune response. Recent studies mainly from our laboratory, assessing the role of the nucleosome in the development of lupus were summarized and put into perspective in **chapter 2** (13).

As outlined above, nucleosomes are probably not immunogenic by themselves. However, apoptosis-induced histone modifications could initiate the anti-nucleosome autoimmune response. In **chapter 3**, we assessed whether apoptosis-induced histone modifications are targets for SLE autoantibodies. In addition, the effect of administering peptides containing apoptosis-induced histone modifications on the development of SLE was evaluated.

Normally, apoptotic cells are directly removed. However, a disturbed apoptosis could lead to the release of apoptotic material into the circulation, including modified histones and nucleosomes. Using an *in vitro* phagocytosis assay, we analysed the phagocytic efficacy of resident peritoneal macrophages for apoptotic cells from pre-diseased and diseased SLE mice (**chapter 4**) (96) .

The release of apoptotic nucleosomes leads eventually to the activation of the immune system and the production of anti-nucleosome autoantibodies (i.e. anti-DNA, anti-histone and nucleosome-specific antibodies). In **chapter 5**, we performed an analysis of the reactivity of anti-nucleosome, anti-DNA, anti-histone and anti-C1q autoantibodies in plasmas of patients from a prospective study on the treatment of proliferative lupus nephritis.

Since specific detection of nucleosome-specific autoantibodies is very difficult sofar, identification of mimotopes for nucleosome-specific autoantibodies could lead to more insight into the significance of this class of autoantibodies. For this purpose we screened random peptide phage display libraries to identify mimotopes for monoclonal nucleosome-specific autoantibodies derived from SLE mice (**chapter 6**) (97). In this study we also evaluated previously identified mimotopes for anti-DNA autoantibodies. In **chapter 7**, the same phage display technique was used to identify the epitope of a SLE-derived anti-histone autoantibody (98). Moreover, the influence of modified residues and amino acid variations between histone variants, residing in the identified epitope, on the binding of this antibody to histone peptides was assessed.

Complexes of anti-nucleosome autoantibody and nucleosomal material (i.e. histones and DNA) can bind to (charged) proteins and peptides which plays a decisive role in the initiation of lupus nephritis. This 'cross-reactivity' may not only lead to false positive reactivity in diagnostic assays, but may also play a role in the development of antigen-spreading in SLE. Therefore, we evaluated the binding of DNA or nucleosomes to the cationic major autoepitope of the SmD1 antigen, i.e. SmD1(83-119) (**chapter 8**).

The research of our group in previous years on the binding of anti-nucleosome/nucleosome complexes to heparan sulfate in the glomerular basement membrane is summarized in **chapter 9**.

Finally, in **chapters 10 and 11** all findings are summarized and put into perspective.

Chapter 2.

Triggers for anti-chromatin autoantibody production in SLE

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Abstract

The formation of autoantibodies against chromatin is the main feature of systemic lupus erythematosis (SLE), an autoimmune disease, which is T-cell dependent and autoantigen-driven. Historically, antibodies against dsDNA, one of the components of chromatin, are considered as a hallmark of SLE. However, dsDNA is poorly immunogenic. Nucleosome-specific T helper cells have been identified. These T cells propagate not only nucleosome-specific antibodies, but also anti-DNA antibodies. Nucleosomes are formed during apoptosis by cleavage of chromatin, and evidence of disturbed apoptosis has been found especially in certain murine models of lupus. In addition to an increased rate of apoptosis, autoimmunity against chromatin might also result from an impaired phagocytosis of apoptotic material, for which strong evidence has been provided by studies in certain knock-out mice (C1q, SAP, DNaseI). The induction of an immune response to nucleosomes could be enhanced by modifications of histones or DNA during apoptosis, altered presentation by antigen presenting cells or a viral infection. The release of nucleosomes and the formation of anti-chromatin autoantibodies result in formation of complexes, which bind to the glomerular basement membrane via heparan sulfate. This deposition incites glomerulonephritis, the most serious manifestation of SLE.

The human cell nucleus contains our genetic information encoded in 2m of double-stranded DNA(dsDNA). These very long strands of dsDNA are packed in the tiny nucleus by a complex folding of higher order chromatin structures, that consist of DNA and numerous proteins. The basic structure of chromatin is the so-called 'beads on a string' fibre. An individual 'bead', a nucleosome, consists of 146 base pairs of dsDNA wrapped around pairs of four different core histones. The core histones are histone H2A, H2B, H3 and H4, while another histone (H1) is bound outside the nucleosome. The string between the beads is formed by linker DNA connecting the individual nucleosomes.

The formation of autoantibodies against chromatin is the main feature of systemic lupus erythematosus (SLE), an autoimmune disease which is T cell dependent and autoantigen-driven.(99) Chromatin autoantibodies have been detected in 88% of SLE patients (64) and have also been found with high incidence in lupus mouse models. Moreover, 50% of the pathogenic T helper cells are nucleosome-specific.(60) It has been reported that in other autoimmune diseases, such as scleroderma and mixed connective tissue disease, anti-chromatin antibodies are also formed (68), but this was not confirmed in another study (70). Autoantibodies against the basic structure of chromatin can be divided in three groups: antibodies reacting with either DNA or histones, and antibodies that have a much higher affinity for the intact nucleosome than the individual components,

DNA and histones. Historically, antibodies against dsDNA are considered as a hallmark of SLE. These antibodies can be detected in 70% of SLE patients, correlate with disease activity, and an increasing titre of anti-DNA precedes exarcerbations (100). Later, it appeared that nucleosome-specific antibodies have an even higher prevalence in SLE and a better correlation with lupus nephritis (70, 101). Since naked DNA is a poor immunogen, it is believed that the nucleosome is the primary antigen, also resulting in the induction of anti-DNA and anti-histone responses via antigen spreading (64).

Nucleosomes are formed during apoptosis by cleavage of the internucleosomal linker DNA. These nucleosomes, probably altered during the apoptotic process, appear with other autoantigens in apoptotic blebs at the surface of apoptotic cells (36). Normally, these apoptotic cells are removed swiftly by phagocytic cells, before apoptotic blebs can be released. Increased presentation of (modified) apoptotic debris and/or impaired removal of apoptotic cells may lead to an immune response against the contents of these blebs (Figure 1).

Furthermore, anti-nucleosome/nucleosome complexes can mediate tissue lesions, since these complexes can bind to negatively charged components in basement membranes (94), such as heparan sulfate. This binding is due to the interaction of positively charged residues in the N-terminal tails of the core histones (102). In particular, anti-DNA and nucleosome specific antibodies are nephritogenic, since they neutralize the negative charges in dsDNA within the nucleosome (82). Deposition of these complexes in the glomerular basement membrane (GBM) incites glomerulonephritis, the most serious manifestation of SLE (Figure 1). The discussion of the characteristics of this nucleosome mediated autoantibody binding to the GBM is beyond the scope of this review. In this article we will focus on the mechanisms that are thought to be involved in the formation of autoantibodies against chromatin/nucleosomes.

Dysregulated apoptosis

Apoptosis is the process of programmed cell death. During apoptosis intracellular structures, including the nucleus, are broken down via certain pathways by specific enzymes. It is specific for apoptosis that chromatin is cleaved into nucleosomes by specific Dnases. This pleads for a possible connection between apoptosis and SLE. Indeed, it now seems clear that a disturbance in the apoptotic process is important in the induction of an anti-chromatin immune response (103).

One of the pathways which leads to apoptosis involves Fas (CD95) and Fas ligand (FasL). Fas and its specific ligand are cell-surface proteins and their interaction triggers a cascade of intracellular events that results in apoptosis of the Fas-expressing targets. In contrast to FasL, which is only expressed on immune cells, expression of Fas is more widespread. Mouse models with abnormalities in the Fas/Fas ligand system initially showed the relation between disturbed apoptosis and SLE. Mutations in the genes encoding Fas or FasL, termed lpr and gld, respectively, give rise to both lymphoproliferation and autoimmune disorders (104-106), which could be prevented by transgenic correction of the respective deficiencies (107). Inhibition of Fas-mediated apoptosis by the injection of soluble Fas into CD1 female mice also resulted in autoimmunity (108). Fas-FasL interactions are involved in the elimination of peripheral anergic or self-reactive B cells. An insufficient elimination could lead to the survival of autoreactive lymphocytes and consequently cause autoimmune phenomena. It is important to note that lpr or gld mutations alone do not cause, but accelerate, lupus-like syndromes, while this depends on the background in which the mutation is generated. Overexpression of Bcl-2, an inhibitor of apoptosis, is another example of disturbed apoptosis leading to autoimmunity. Here, the overexpression leads to an increased number of autoreactive B cells and autoimmunity (109). Altogether, these mouse models indicate that a disturbed apoptosis can lead to SLE.

In humans, however, the evidence for a disturbed apoptosis is not so clear. Patients with a defect in the Fas molecule or Fas-ligand develop a non-malignant lymphoproliferation accompanied by haemolytic anaemia, the so called autoimmune lymphoproliferation syndrome (ALPS) (110). Only a quarter of the patients develop anti-nuclear autoantibodies. In humans with SLE, the Fas-dependent pathway appears to be unaffected (110). However, some evidence points to a dysregulation of apoptosis at another level. In SLE patients, the soluble form of Fas appeared to be upregulated (111, 112), although others report no or little evidence of any elevation in serum levels of soluble Fas (113-115). In addition, Bcl-2 expression was increased (116) and the fraction of peripheral blood lymphocytes expressing Fas was increased (117). Moreover, elevated levels of apoptotic cells (47, 48) and an increased rate of *in vitro* apoptosis of mononuclear blood cells (118, 119) were found. However, these results did not always correlate with disease activity and similar data have been obtained in non-SLE patients. If the rate of apoptosis really is increased in patients with lupus this phenomenon may be explained by an elevated number of circulating lymphocytes prone to apoptosis. In conclusion, unlike murine models of lupus, evidence of disturbed apoptosis in patients with SLE is somewhat conflicting and thus its contribution to the pathogenesis of the disease has to be clarified.

Disposal of apoptotic material

In addition to an increased rate of apoptosis, as described above, elevated levels of nucleosomes might result from an impaired phagocytosis of apoptotic material. The linkage between the source of autoantigens and apoptosis made us (11) and others (120) believe that a defective or impaired phagocytosis of apoptotic cells underlies the generation of autoantibodies in SLE. Rosen et al. described autoantigens, such as nucleosomes, becoming clustered in blebs on the surface of apoptotic keratinocytes after exposure to UV light (36). These autoantigens may become extra immunogenic, since they can be modified by several processes operative during apoptosis (detailed below), so it is important that clearance is fast and efficient. Normally, apoptotic cells are recognized by phagocytic cells at an early stage of apoptosis. Surface changes on the apoptotic cell, such as flipping of phosphadityl serine (PS) from the inside to the outside of the cell membrane, mark the cell for clearance. Several receptors on the phagocyte are involved in the recognition of apoptotic cells, but the whole process is far from clear at this moment (121).

In addition to a direct contact of receptor and ligand, a role for mediators has been described. These are molecules like C1q, b2- glycoprotein I, thrombospondin, C-reactive protein (CRP) and serum amyloid P (SAP). Their relevance for an adequate removal of apoptotic cells is underlined by several high-impact studies using knock-out mice. These studies are summarized in Table I. C1q can bind directly and specifically to surface blebs of apoptotic human keratinocytes *in vitro* and *in vivo* (122, 123), thereby facilitating clearance of apoptotic material. Homozygous deficiency of C1q in mice (129.C57Bl/6) resulted in the development of antinuclear antibodies and glomerulonephritis in 25% of the mice at 8 months of age (124). These mice also showed an increase in apoptotic bodies in the glomerulus. Indeed, clearance of apoptotic cells after i.p. inoculation was less efficient in these C1q-deficient mice (125). However, no difference in the rate of clearance of apoptotic keratinocytes could be observed between C1q knock-out and wild-type mice (126). Thus, this suggests that C1q is not required for the clearance of apoptotic keratinocytes. Deficiency of C1q alone is not sufficient for development of autoimmunity, since other genetic factors are also needed. In these C1q deficient mice the background is of great importance. Mice with the disrupted C1q gene were backcrossed for seven generations into C57Bl/6 and MRL/+ strains. This MRL/+ strain is the background of the earlier mentioned lpr (Fas) mutation, which develops autoimmunity at a later age than MRL/lpr mice. Fas and/or C1q-deficient C57Bl/6 mice showed absolutely no autoimmunity, but in C1q-deficient MRL/+ mice an acceleration of autoimmunity was observed (126). Disease was particularly pronounced in female mice, as in human SLE patients. Only a minor

Table I. Prevalence of anti-nuclear autoantibodies and glomerulonephritis in mice with a targeted disruption of molecules relevant for phagocytosis of apoptotic cells.

Deficient molecule[a]		Anti-nuclear autoantibody formation[b] (% of positive mice)				Glomerulonephritis[c] (% of positive mice)	Reference
		ANA	anti-histone	anti-dsDNA	anti-nucleosome		
(129xC57Bl/6).C1q		43	43	ND[d]	ND	25	(124)
C57Bl/6.C1q		0	ND	ND	ND	0	(126)
MRL/+.C1q		83	ND	ND	ND	33	(126)
SAP	(M)[e]	82	54	46	74	42	(129)
	(F)	63	31	25	59	4	(129)
	(M/F)	96	ND	ND	ND	0	(130)
DNase I	(M)	73	25	38	88	31	(131)
	(F)	56	24	12	71	19	(131)
IgM		ND	ND	70	ND	70	(132)
mer^{kd}		ND	ND	58	ND	ND	(133)

[a] Except for mer^{kd} mice, deficiency was induced by targeted disruption of the gene coding for the listed molecule. Mer^{kd} mice have a cytoplasmic truncation of the Mer receptor induced by targeted disruption.
[b] Several mouse strains were used for these knock-out experiments. In certain wild-type strains a low prevalence of autoantibodies was also present. For details see the respective references.
[c] Glomerulonephritis is defined as either histological evidence of glomerulonephritis and/or the presence of immunoglobulin and/or C3 deposits.
[d] ND, not determined.
[e] M, male; F, female.

decrease of *in vivo* clearance of apoptotic cells by activated macrophages was found. In humans a C1q-deficiency is strongly associated with SLE. Monocyte-derived macrophages of a C1q-deficient patient displayed a defective clearance of apoptotic Jurkat T cells *in vitro* . Purified C1q could correct this in a dose-dependent manner. Antibodies to C1q have been detected in lupus mice (127) and SLE patients, which correlated with disease activity and nephritis (128). These antibodies could block the protective function of C1q, by preventing binding of C1q to apoptotic cells.

Beside C1q, priming of apoptotic cells for phagocytosis is also mediated by serum IgM. Mice deficient for serum IgM developed an autoimmune response and glomerular deposits after 12-18 months, but no glomerulonephritis was observed (134). When secretion of IgM was blocked in MRL/lpr mice an enhanced

production of IgG antibodies to dsDNA occurred, with more abundant deposits of immune complexes in the glomeruli leading to a more severe form of glomerulonephritis (132). However, selective IgM deficiency in humans has been described only rarely and is not associated with systemic autoimmunity. Serum amyloid P (SAP) is another mediator related to the removal of apoptotic cells. SAP binds *in vivo* to apoptotic cells, apoptotic blebs and chromatin, thereby preventing recognition by the immune system of these autoantigens. SAP also provides a clearance mechanism through opsonization by polymorphonuclear leukocytes (PMN), since it also binds to Fcγ-receptors (135). SAP-deficient mice develop an anti-chromatin response, severe glomerulo-nephritis at 8 months in 4% of the male and 42% of the female mice (129). However, another study showed that SAP-deficient mice had high anti-chromatin antibody titres, but no severe glomerulonephritis (130). This latter study suggested an important role for SAP in preventing LPS toxicity rather than autoimmunity. SAP-deficient mice also showed an enhanced anti-chromatin response upon immunization with chromatin from chicken erythrocytes. These findings indicate that SAP plays an important role in the regulation of chromatin disposal. Indeed, the presence of SAP/DNA complexes is lower in SLE patients compared with healthy controls, especially in patients with high anti-DNA antibody titres (136). SLE patients have a normal level of circulating SAP, but CRP, functionally related to SAP, is decreased during flares in SLE (137).

In addition to processes that protect or prime chromatin for disposal, impaired degradation of chromatin is another process that may be involved in the developmentof SLE. DNaseI is an important enzyme involved in the degradation of chromatin by cleavage of dsDNA. It is also the major nuclease in serum and is present at sites of high cell turnover. Mice deficient for DNaseI showed an anti-chromatin antibody response and glomerulonephritis at 8 months (131). This was more evident in female (31%) than in male (19%) mice. Recently, a mutation in exon 2 of the DNaseI gene was found in 2 SLE patients (138). These patients showed an extremely high titre of IgG antibodies against nucleosomal antigens. It is known that DNaseI activity is decreased in murine and human lupus (139), but treatment with recombinant DNaseI failed to attenuate glomerulonephritis (140). These findings suggest an important role for DNaseI in the development of anti-chromatin responses in SLE. Moreover, DNaseI is involved in the induction of apoptosis by cleavage of chromatin into nucleosomes, as was described above.

Very recently, the involvement of a new receptor for the clearance of apoptotic cells has been described. Macrophages of knock-out mice with a truncated cytoplasmic tail of the Mer receptor tyrosine kinase (mer^{kd}) showed a defect in the clearance of apoptotic cells (133). Earlier it has been shown that the protein encoded by growth arrest-specific gene 6 (GAS6), a ligand for the Mer receptor, mediated the binding of phosphatidyl serine to macrophages (141). Mice with a defective Mer

receptor, which were injected with dexamethasone to induce apoptosis of cortical thymocytes, showed a seven-fold increase in the number of apoptotic cells compared with wild-type mice. A difference in the rate of apoptosis was eliminated as an explanation. Moreover, injection of fluorescent-labeled apoptotic thymocytes intraperitoneally or intravenously confirmed that mice with the abnormal Mer receptor could not adequately phagocytose these cells. The impairment of phagocytosis was not due to an inability to bind apoptotic cells, because binding did not differ between wild-type and Mer-deficient macrophages, but only macrophages from wild-type mice were able to ingest apoptotic thymocytes. Interestingly, more than 50% of the mer^{kd} mice showed autoimmunity against DNA at 4-5 months of age (similar to MRL/lpr mice) (133). Macrophages of SLE patients have been shown to have a decreased ability to phagocytose apoptotic cells (44). Instead of being a cause, this can also be a secondary effect of the disease, because nucleosomes (52) and autoantibodies can inhibit the binding of apoptotic cells to phagocytes. We analysed the phagocytosis in premorbid SLE mice with an newly developed assay (142). No constitutive defect could be found in these mice (42), however in lupus mice phagocytosis was impaired (96).

In conclusion, these studies in knock-out mice provide strong evidence for the contribution of a disturbed disposal of apoptotic cells in the triggering of an anti-chromatin response, and the subsequent development of glomerulonephritis.

Modification of autoantigens

Disturbances in apoptosis or phagocytosis of apoptotic cells are a plausible cause for an increased release of apoptotic material. Still, self-antigens are released, which should not be recognized by the immune system. An explanation for the development of autoantibodies could be modifications on these autoantigens during apoptosis. Although tolerance may be intact for the unmodified autoantigen, the modified antigens may fail to tolerize T cells.

Various modifications on known autoantigens have been described to occur specifically during apoptosis (16, 143). Importantly, chromatin is also susceptible to modifying enzymes. Most of these modifications are related to the functions of chromatin in the regulation of transcription, but several have also been shown to occur during apoptosis. Constituents of chromatin, proteins and dsDNA can be modified. Histone acetylation has an important role in maintaining chromatin transcriptional activity, but also in DNA replication and DNA repair. Deacetylation is linked to transcriptionally inactive chromatin parts. The core histones are reversibly acetylated at specific lysine residues located in the N-terminal tail domains, and specific acetylation patterns may have a specific function.

Involvement of histone hyperacetylation in the triggering of apoptosis has been described (23), in contrast to deacetylation of chromatin, which occurs during inactivation of transcription. Phosphorylation of histones plays an important role in regulation of transcription and has also been linked to apoptosis (144). The core histones and H1 undergo phosphorylation at specific serine and threonine residues. In particular, histone H3 is known to contain several sites which can be phosphorylated. Phosphorylation of serines 10 and 28 on histone H3 has been coupled to mitotic chromatin condensation (145). Histone H2B (22) and H1 (30, 144) have been shown to be (de)phosphorylated during apoptosis. Interestingly, other phosphorylated or dephosphorylated intracellular proteins have been shown to be specifically recognized by sera from SLE patients (143, 146). Indeed, immunoprecipitation of ^{32}P-labeled apoptotic Jurkat cells using sera derived from SLE patients revealed different proteins, depending on the patient. In some patients proteins between 12 and 17 kD were also found, which could point to the presence of phosphorylated histones. Histone methylation has different functions that range from transcriptional regulation to the higher-order packaging of chromatin. Histone methylation can occur on arginine or lysine residues, at specific positions in the N-termini of histones H3 and H4. Thus, methylation joins acetylation and phosphorylation as a third component of a 'histone code' that modifies the chromatin structure. Methylation of histones has not yet been reported during apoptosis.

In addition to these three described modifications, other protein modifications such as citrullination, transglutaminase cross-linking and poly(ADP-ribosyl)ation may be important. Differences in the state of poly(ADP-ribosyl)ation have been described in relation to apoptosis (29). Autoimmunity to ubiquitin or ubiquitinated proteins has been reported in SLE (147, 148). A ubiquitin molecule is attached to the C-termini of histone H2A and H2B (148). Another described modification related to autoimmunity is citrullination, a posttranslationally modified arginine. Citrullin is specifically recognized by autoantibodies from rheumatoid arthritis patients (149). The expression of transglutaminase, which crosslinks proteins, has been observed during apoptosis. The role of crosslinking here may be the stabilization of apoptotic bodies, thus preventing the leakage of their content. Substrates of transglutaminase targeted in autoimmune disorders include histone H2B (150). Besides histones, DNA is also modified during apoptosis, including abnormal DNA methylation and CG contents which make DNA more immunogenic (34). Exposure to reactive oxygen species (ROS) or UV-light may also induce immunogenic DNA modifications (151).

Caspases cleave several proteins that are necessary for the execution of apoptosis. The cleavage products are often targets for lupus autoantibodies and are in most cases better recognized than the original protein (152). Recently, granzyme

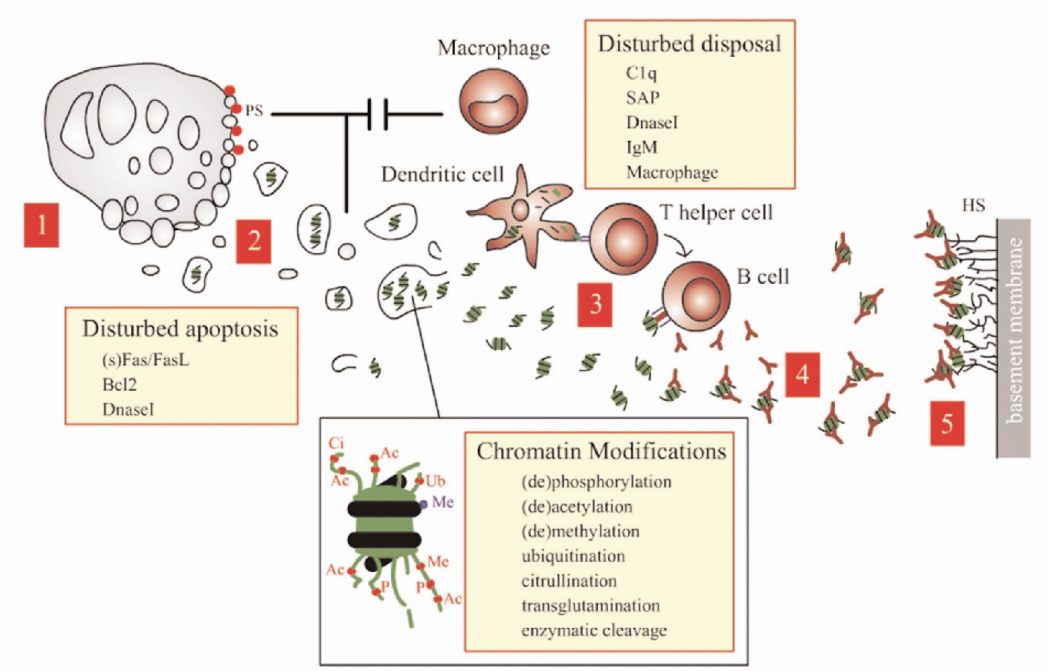

Figure 1. Hypothesis for the development of anti-chromatin responses in systemic lupus erythematosus. (1) Disturbed apoptosis either at the wrong timepoint and/or at the wrong micro-environment; (2) defective removal of apoptotic cells leading to the release of apoptotic blebs containing chromatin; (3) stimulation of immune response by chromatin; (4) formation of anti-chromatin/chromatin complexes; (5) binding of these complexes to heparan sulfate in basal membranes due to interaction between the cationic N-termini of the core histones and the anionic heparan sulfate.

B, one of the proteases involved in apoptosis induced by cytotoxic T cells, was found to be involved. The susceptibility of autoantigens to cleavage in unique fragments by granzyme B may be predictive for a protein to become an autoantigen (16). The precise role of chromatin modifications in the induction of an anti-chromatin autoimmune response remains to be investigated further, as has been found for non-nucleosomal chromatin-proteins (153).

Immunogenicity of chromatin

Although dsDNA often is regarded as the major autoantigen in SLE, *in vivo* DNA has only been found complexed to histones in human and murine lupus (49). T helper cells specific for chromatin, especially histones, have been detected in human and murine lupus (55, 56), which also indicates that nucleosomes rather than dsDNA were driving the autoimmune response in SLE. Indeed, these nucleosome-

specific T cells can also induce autoantibodies to dsDNA and chromatin by epitope spreading (54). Antibodies which react specifically with nucleosomes, not with DNA or histones alone, have been detected in lupus mice(84, 154) and patients (64, 65). Formation of nucleosome-specific antibodies appeared to precede the anti-DNA and anti-histone response (84, 154). Most SLE patients that have nucleosome-specific antibodies also have anti-DNA and anti-histone antibodies. Nucleosome-specific antibodies without other anti-chromatin antibodies have been found in 16-30% of the patients. Although there are at present no large prospective analyses available on the diagnostic accuracy of anti-nucleosome antibodies, in two recent retrospective studies a good correlation was found with disease activity and the occurrence of lupus nephritis (12, 70). There was even a claim that anti-nucleosome reactivity was a better marker than anti-DNA (70). Normally, uptake of apoptotic cells induces an anti-inflammatory response of macrophages (155). Efficient engulfment of apoptotic cells, prior to their lysis, is critical for the suppression of inflammation. When apoptotic cells are recognized by macrophages, production of proinflamatory mediators is downregulated. Such anti-inflammatory mediators profoundly influence whether an immune response will be activated.

Dentritic cells (DCs) are also able to phagocytose apoptotic cells, although less efficiently than macrophages. The DCs can probably mature and present the autoantigens derived from apoptotic cells. So, delayed clearance of apoptotic cells may result in uptake by DCs and presentation to T cells. Modified forms of autoantigen might enhance the response of the immune system after presentation on DCs. Beside presentation of modified forms of nucleosomal peptides, a defective function of antigen-presenting cells could be involved. Abnormalities in APC surface membrane molecules, such as B7 and IgG Fc-receptors, were found in SLE (156, 157). Breakdown of selftolerance may be enhanced by viral infections. The relation between SLE and viral infection has been shown in various studies (158, 159). In addition, the viral T antigen of polyomavirus BK could bind to mammalian DNA and enhanced immunogenicity (160). Viral antigens also become clustered in apoptotic blebs together with chromatin and other autoantigens (161). In conclusion, chromatin is the major autoantigen in SLE, and its immunogenicity might be enhanced by chromatin modifications, defective presentation by antigen-presenting cells or viral infections.

Conclusion

The importance of chromatin and anti-chromatin autoantibodies for SLE has been proven by the detection of nucleosome-specific T helper cells and nucleosome-specific autoantibodies. Importantly, nucleosome/anti-nucleosome complexes can bind to the glomerular basement membrane, which induces lupus nephritis, the most serious disease manifestation in SLE (102).The trigger for the production of anti-chromatin autoantibodies remains to be discovered and is most likely multi-factorial, including genetic susceptibility. The genetic basis of SLE has been investigated by several studies. In mice several genes have been linked to the susceptibility to develop SLE. In the NZW/NZB-related NZM2410 lupus-prone strain three recessive loci (Sle1, Sle2 and Sle3) have been strongly associated with SLE-susceptibility (162). Characterization of strains containing one of these susceptibility loci resulted in the linking of Sle1 with the loss of tolerance to nuclear antigens, especially for the H2A-H2B/DNA subnucleosome particle. Sle2 lowers the threshold of B cells and Sle3 mediates T cell dysregulation. Studies of combinations of these loci have shown that loss of tolerance to chromatin mediated by Sle1 is essential for development of disease. The co-expression of Sle1, Sle2 and Sle3 resulted in systemic autoimmunity and fatal glomerulonephritis (163).

The exclusive formation of nucleosomes in apoptosis has put the focus on the relation between apoptosis and SLE. Mouse models with an abnormal apoptotic process (MRL/lpr, gld) develop lupus-like disease features. Recently, several studies with mouse models deficient for molecules involved in the removal of apoptotic cells have proven the importance of this process for the induction of autoimmunity. Most of these mice developed an anti-chromatin response and sometimes glomerulonephritis. In addition to a defect in the removal of chromatin, modifications of histones or DNA induced during apoptosis, altered presentation by APCs or a viral infection might increase the chance that the immune system develops a response to chromatin. Since SLE is a multi-factorial disease, even more factors may be involved and most likely a cascade of functions needs to be altered. More research has to be performed to identify the finger that pulls the trigger.

Chapter 3.

Apoptosis-induced acetylation of histones is pathogenic in lupus

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Submitted

Abstract

Systemic lupus erythematosus (SLE) is an autoantigen driven and T cell-dependent autoimmune disease, in which the nucleosome has been identified as a major autoantigen. In lupus an inadequate removal of apoptotic cells may lead to the challenge of the immune system with immunogenic self-antigens that have been modified during apoptosis. Using different approaches, we demonstrated that a lupus-derived monoclonal antibody specifically recognizes apoptosis-induced acetylation of histones H4 and H2A. In addition, plasmas from both lupus patients and mice showed a higher reactivity with hyperacetylated histones extracted from cells treated with a histone deacetylase inhibitor or an apoptosis inducer. Importantly, administration of a histone H4 peptide containing the identified apoptosis-induced acetylation motif to pre-diseased lupus-prone mice accelerated the disease, and aggravated mortality, proteinuria, skin lesions and glomerular IgG depositions. Administration of the corresponding non-acetylated histone H4 peptide had no pathogenic effect. Furthermore, the delayed type hypersensitivity response in lupus mice against the tri-acetylated histone H4 peptide was higher than against the non-acetylated histone H4 peptide, while BALB/c mice did not show a response. Therefore, we conclude that apoptosis-induced histone acetylation represents an important driving force in the autoimmune response in lupus.

The formation of autoantibodies against chromatin components (i.e. DNA, histones, and nucleosomes) is a major feature of systemic lupus erythematosus (SLE), an autoantigen driven, T cell-dependent autoimmune disease that affects millions of people world-wide. In SLE, the nucleosome is a major autoantigen, and nucleosome and histone-specific autoreactive T and B cells have been identified (64). Years before clinical manifestation of the disease anti-nuclear antibodies can be detected in the circulation of future SLE patients (164). Anti-chromatin autoantibodies in complex with chromatin are targeted to basement membranes, including the dermal and the glomerular basement membrane. The deposition of chromatin-containing immune complexes incites glomerular inflammation, which is one of the most serious clinical manifestations of SLE (82, 94, 99, 165).

Nucleosomes can be detected in the circulation during disease in lupus patients and mice (49-51). There is evidence that in lupus either an aberrant apoptosis or a reduced clearance of apoptotic cells, or a combination of both, leads to an overflow of the cleaning machinery and the presence of apoptotic chromatin in the circulation (39, 40). The contribution of an aberrant apoptosis for the development of SLE is supported by mouse models with an abnormal function of

factors involved in apoptosis. Deficiency of Fas (lpr), FasL (gld), Bcl2/Bim, PDCD1, PTEN, BLyS, and TACI leads to the formation of anti-nuclear antibodies and glomerulonephritis (105, 106, 109, 166-170). In addition, an impaired removal of apoptotic cells also predisposes to the development of SLE. Mice deficient in factors required for a proper clearance of apoptotic cells such as DNaseI, SAP/CRP, C1q, IgM and Mer develop anti-nucleosome autoantibodies and glomerulonephritis (40, 124, 129, 131, 133, 134). Most importantly, it has been demonstrated that the clearance of apoptotic material by phagocytes is impaired in both lupus patients and mice (44, 96).

Autoantigens targeted in SLE are clustered in blebs at the surface of late apoptotic cells (36, 37). Autoantigens may contain apoptosis-induced modifications that constitute epitopes that normally are not encountered by the immune system when clearance of apoptotic cells is adequate. Apoptosis-induced autoantigen modifications include cleavage by caspases, endonucleases or granzyme B (16, 17). Furthermore, autoantigens may be modified by the addition or removal of covalently linked moieties through acetylation, methylation, phosphorylation, ubiquitination, citrullination, ADP-ribosylation or transglutamination (17, 290). These covalently linked modifications have also been reported for nucleosomes/histones, but not exclusively and uniformly in the context of apoptosis, and primarily in the context of regulation of transcription (291). Although it has been demonstrated that apoptotic cells may induce a lupus-like immune response, nothing is known about apoptosis-induced chromatin modifications as targets for autoantibodies in SLE (58, 171-173).

We show here that a monoclonal lupus-derived autoantibody specifically recognizes histone H4 peptides acetylated at lysine residues 8 and/or 12 and/or 16. Furthermore, plasmas from lupus mice and patients showed a higher reactivity with acetylated H4 peptides and hyperacetylated histones extracted from cells that were treated with either a histone deacetylase inhibitor or an apoptosis inducer. Most importantly, administration of a H4 peptide tri-acetylated at lysine residues 8, 12 and 16 accelerated disease development in MRL/lpr mice, while administration of a non-acetylated H4 peptide had no effect. Finally, in lupus mice the delayed type hypersensitivity response against the tri-acetylated H4 peptide was higher than against the non-acetylated H4 peptide.

Materials & Methods

Cell culture, inhibition of histone deacetylases and induction of apoptosis.

Jurkat or U937 cells were routinely cultured. To inhibit histone deacetylase activity, cells, at a concentration of $4*10^5$ cells/ml, were treated with 100 ng/ml TSA (Biomol) for 16 hours. To induce apoptosis, cells, at a concentration of $2*10^5$/ml were treated with 2 µg/ml camptothecin (CPT) (Sigma) or anti-Fas antibody diluted 1:1000 (Sigma) for the indicated periods of time. The progress of apoptosis was determined by analyzing AnnexinV-FITC binding and Propidium Iodide staining with flow cytometry.

Antibodies and random peptide phage display epitope mapping.

KM-2 is a monoclonal anti-histone autoantibody derived from the MRL/lpr lupus mouse, which recognizes the N-terminal tails of histone H2A and histone H4 (95). The exact epitope of KM-2 was determined by screening a 15-mer random peptide phage display library (New England Biolabs) according to the manufacturer's instructions. Other antibodies used in this study were #34, which solely recognizes unmodified histone H3.1/H3.2 (98), and antibodies specific for histone H4 acetylated at lysine residues 5, 8, 12 and/or 16, histone H2A acetylated at lysine residues 5 or 9, histone H2B phosphorylated at serine residue 14, and CBP (Upstate), actin, caspase-3, PCAF, HDAC1 and p300 (Santa Cruz).

Peptide, dsDNA and nucleosome ELISA.

Peptides used in this study were either synthesized by the peptide synthesis facility of the CNRS in Strasbourg or commercially obtained (Eurosequence B.V.) and in all cases analyzed for integrity and purity by HPLC and mass spectrometry. The reactivity of KM-2 (starting at a concentration of 5 µg/ml) and plasmas from both MRL/lpr lupus mice and SLE patients (diluted 1:100) with coated peptides in ELISA was tested as described previously (97). The use of plasma from SLE patients was approved by the local ethical committee of the Radboud University Nijmegen Medical Centre. For detection, the appropriate horseradish peroxidase-conjugated antibody (Southern Biotechnology Associates) was used. For the peptide inhibition ELISA, KM-2 was incubated in a 96-wells plate with different concentrations of inhibiting peptides for 1 hour and then transferred to Maxisorb® 96-well plates (NUNC) coated with 2 µM non-acetylated histone H4 peptide and incubated for 1 hour and an appropriate horseradish peroxidase-conjugated antibody

was used. Histone, dsDNA and nucleosome ELISA were performed as described previously (97).

Preparation of cell extracts, extraction of histones, and protein determination.

For analysis of histones, total cell extracts were prepared directly from PBS-washed and pelleted cells ($2*10^5$) in 250 µl Laemmli buffer followed by boiling for 5 min. Alternatively, total cell extracts were prepared by lysing PBS-washed and pelleted cells ($2*10^5$) in 250µl lysis buffer (250 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol (DTT), 50 mM HEPES pH 7.0, 5 mM EDTA) containing a cocktail of protease inhibitors (Roche) and sonicated on ice two times with bursts of 15 s. Subsequently, histones were extracted with 0.2 M H_2SO_4, precipitated with 20% TCA and washed with ice-cold, acidified (0.1% HCl) acetone and the pellet was dissolved in 100 µl of 100 mM Tris.HCl pH 8.0. For histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity assays, total cell extracts were prepared in ELB lysis buffer (without DTT), sonicated on ice two times with burst of 15 s, and cellular debris was removed by centrifugation at 14,000 x g at 4°C for 10 min. Protein concentration was determined by the Bicinchoninic acid assay (Sigma) with BSA as a standard.

SDS-PAGE, Western blotting and immunofluorescence staining of cells.

Proteins resolved by SDS-PAGE were transferred to nitrocellulose blots and were probed with the indicated primary antibody. Blots were incubated with an appropriate horseradish peroxidase-conjugated antibody (Southern Biotechnology Associates). Finally, blots were developed using ECL (Amersham) and exposed to Hyperfilm ECL X-ray films (Amersham). Cytospinned cells were fixed with 2% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Cells were incubated with the indicated primary antibodies and an appropriate Alexa-488 conjugated antibody (Molecular Probes) was used. DAPI and TUNEL (Roche) staining were performed according to the manufacturer's instructions.

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity assays.

HAT activity was measured using the HAT activity Assay Kit (Biovision) and HDAC activity was measured using the HDAC Fluorescent Activity Assay Kit (Biomol) according to the manufacturer's protocols.

Mice and evaluation of albuminuria, DTH and IgG deposition.

Eight-weeks-old MRL/lpr or BALB/c mice (Jackson Laboratory) were injected subcutaneously with non-acetylated histone H4 peptide (residues 1-22), the same histone H4 peptide tri-acetylated at lysine residues 8, 12 and 16, or PBS. Mice received 100 μg peptide or PBS emulsified in CFA once and three booster injections with 100 μg in IFA at 2 week intervals. Plasmas and urine were collected weekly. Albuminuria was tested using Albustix (Bayer). Delayed type hypersensitivity (DTH) was performed as described (174). Glomerular IgG depositions were visualized in 2 μm frozen section of diseased mice by immunofluorescent staining with Alexa-488 conjugated anti-mouse IgG antibody. For each group 50 glomeruli were blindly scored on a scale from 0 to 3 by three independent observers. Experimental handling of the animals was approved by the local animal ethical committee of the Radboud University Nijmegen Medical Centre.

Results

Identification of the epitope for the lupus-derived monoclonal autoantibody KM-2

According to our hypothesis apoptosis-induced post-translational modifications of histones may play an important role in the initial immune response against chromatin in lupus. Therefore, we started to map the epitope of the lupus-derived mouse monoclonal autoantibody KM-2. Previously, we showed that KM-2 reacts in ELISA with both a histone H4 peptide (residues 1-29) and a histone H2A peptide (residues 1-20), which are very similar in amino acid sequence (95). KM-2 also reacts with whole histone H4 and histone H2A. Here, we applied the random peptide phage display method to map precisely the epitope of KM-2, which yielded the amino acid sequence RGGLGLVNGRSLRH. Amino acid sequence analysis revealed 71.4% similarity with histone H4 (i.e. residues 5-KGGKGLGKGGAKRH-18) and a much lower similarity of 42.8% with histone H2A (i.e. residues 6-QGGKARAKAKSRSS-19). However, the positively charged lysine residues at positions 8, 12 and 16 (K8, K12 and K16), which are normally present in histone H4, could not be found in the identified motif. This result suggested that in the epitope for KM-2 these lysine residues could be modified, i.e. acetylated, and therefore were not identified by the random peptide phage display technique, which makes use of a bacterial host not capable of histone acetylation by definition. Therefore, the reactivity of KM-2 was determined with synthetic histone H4 peptides (residues 1-22), either non-acetylated or acetylated at K5, K8, K12 and/or K16, directly coated in ELISA (Fig. 1A). Compared to the non-acetylated histone H4 peptide, KM-2 showed an enhanced reactivity with histone H4 peptides acetylated at K8, K12 and/or K16, while the acetylation of K5 did prevent the binding of KM-2. In addition, an inhibition ELISA with competitor peptides in solution was performed to rule out possible differences in coating efficiency. This revealed that the reactivity of KM-2 with the non-acetylated histone H4 peptide was strongly inhibited by histone H4 peptides acetylated at K8, K12 and/or K16, while histone H4 peptides that were acetylated at K5 did not inhibit KM-2 binding (Fig. 1B).

To test whether KM-2 could also react with acetylated histone H4 in situ we stained Jurkat cells that were either untreated or treated with Trichostatin A (TSA), which is a specific histone deacetylase (HDAC) inhibitor that results in hyperacetylation of histones. Staining of untreated cells with KM-2 revealed a weak, fine granular nuclear staining that clearly increased after TSA treatment. A similar

result was obtained by staining with commercially available antibodies that are specific for histone H4 acetylated at K8 and K12, i.e. anti-H4-AcK8 and anti-H4-AcK12 (Fig. 1C). In addition, acid-extracted histones derived from TSA-treated Jurkat cells were resolved and blots were probed with KM-2, anti-H4-AcK8 and anti-H4-AcK12 antibodies. TSA treatment strongly enhanced the reactivity of all these antibodies with histone H4. As expected, using anti-H4-AcK5 and anti-H4-AcK16 gave similar results (unpublished data). In addition to histone H4, the reactivity of KM-2 with histone H2A was increased upon TSA treatment. Apparently, the region within H2A that is homologous to the identified epitope motif and the N-terminal region of histone H4 includes one or more residues that can be acetylated (Fig. 1D).

Taken these data together, the primary epitope for the monoclonal lupus-derived autoantibody KM-2 is located within the N-terminal tail of histone H4 and includes acetylated lysine residues at position 8 and/or 12 and/or 16.

Lupus-derived autoantibodies recognize apoptosis-induced histone modifications

Since apoptosis-induced histone modifications may be the targets for lupus autoantibodies, the reactivity of KM-2 was tested with equally loaded histones extracted from both normal and apoptotic Jurkat cells. Apoptosis was induced by 4 hours incubation with camptothecin (CPT), a DNA topoisomerase I inhibitor. Cells were either directly dissolved in Laemmli sample buffer or extracted by sulphuric acid to ensure that all histones were efficiently extracted and recovered for analysis. Induction of apoptosis clearly enhanced the reactivity of KM-2 with both histone H4 and histone H2A (Fig. 2A). Commercially available antibodies that are specific for different acetylated forms of histone H4 or histone H2A also showed an enhanced reactivity with histone H4 or histone H2A derived from apoptotic cells. As a control we included an antibody specific for histone H2B phosphorylated at serine residue 14, which is a well-established apoptosis-induced histone modification that is also associated with double stranded DNA breaks (18, 292). Indeed, this latter antibody also showed an enhanced reactivity with apoptotic histone H2B. On the other hand, the reactivity of another monoclonal lupus-derived autoantibody, which is specific for unmodified histone H3.1/3.2 variants (98), was not different for normal and apoptotic cells (Fig. 2A). Immunofluorescent double staining of both normal and apoptotic Jurkat cells by KM-2 and TUNEL, which stains for apoptotic DNA fragmentation, clearly revealed co-staining, especially of apoptotic blebs (Fig. 2B). The reactivity of KM-2 was also analyzed with histone H4 and histone H2A extracted from normal and Jurkat cells that were apoptotic for different time periods.

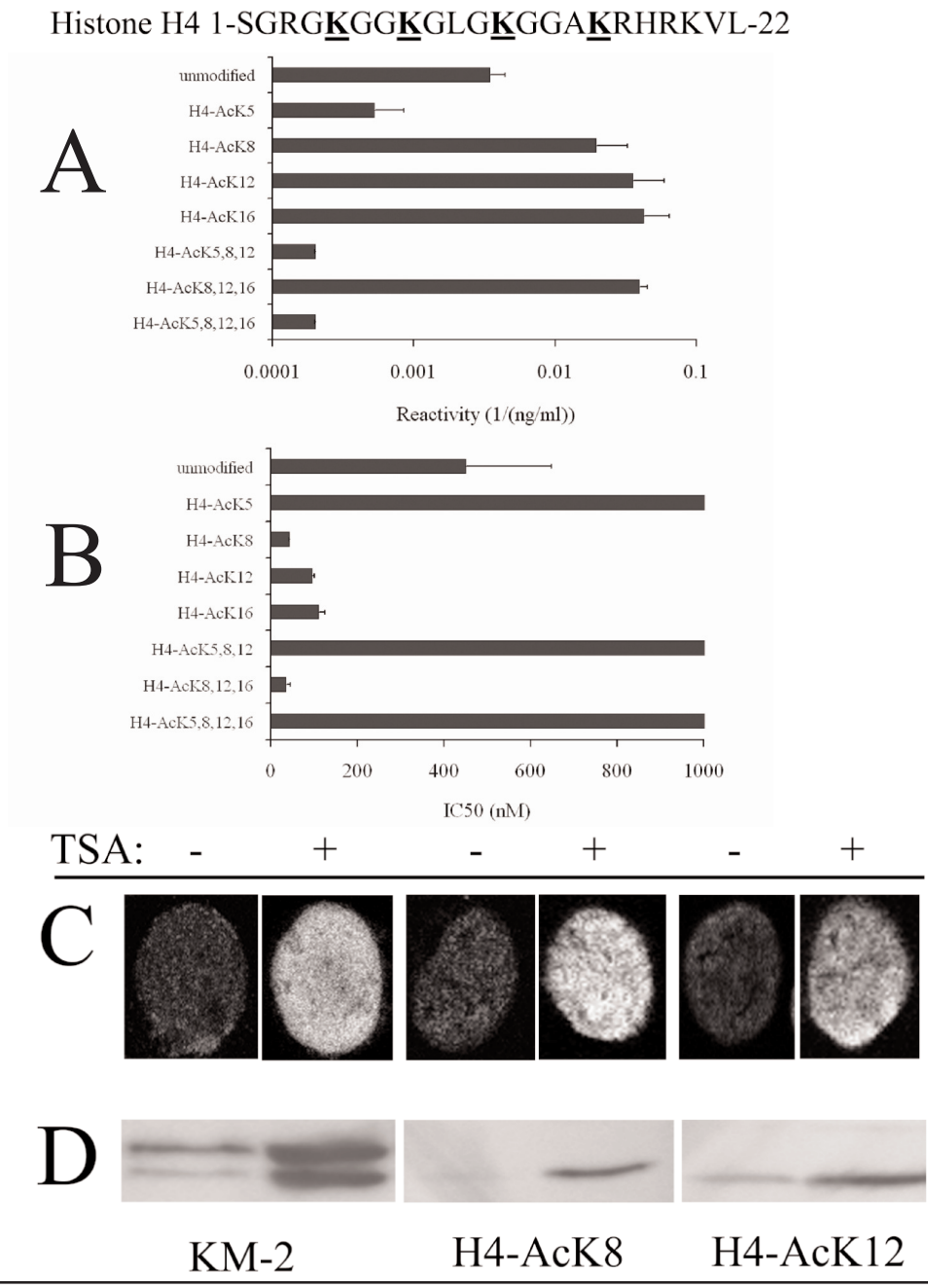

Figure 1. The lupus-derived autoantibody KM-2 recognizes acetylated histone H4. **A.** Reactivity of KM-2 with acetylated H4 peptides (residues 1-22). **B.** Inhibition of binding of KM-2 to non-acetylated H4 peptide by acetylated H4 peptides. **C.** Enhanced staining of TSA-treated Jurkat cells by KM-2, anti-H4K8Ac and anti-H4K12Ac, respectively. **D.** Enhanced reactivity of KM-2, anti-H4-AcK8 and anti-H4-AcK12 with acid extracted and equally loaded histones from TSA-treated Jurkat cells. An antibody specific for actin was used as an internal loading control.

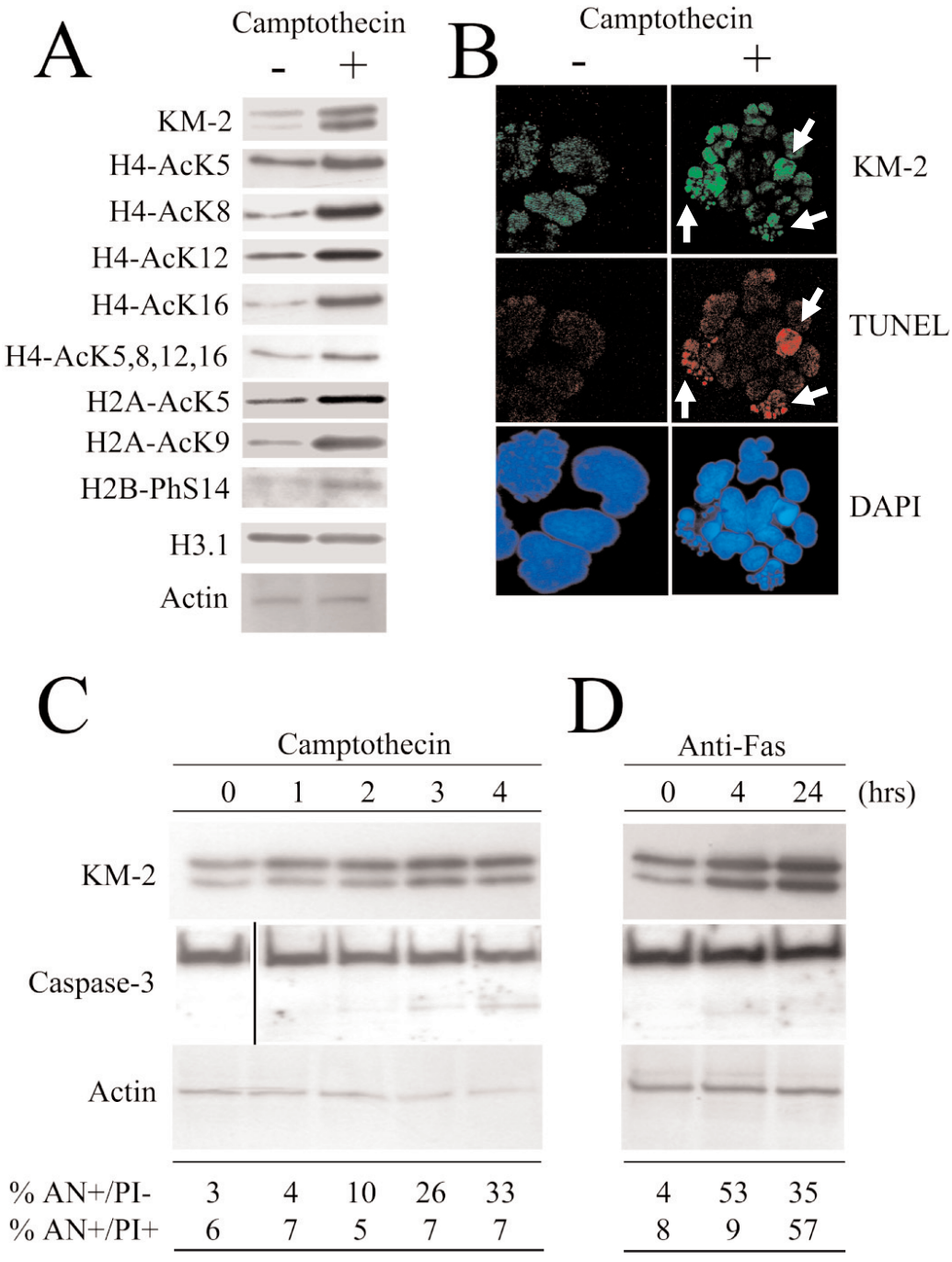

Figure 2. The lupus-derived autoantibody KM2 recognizes apoptosis-induced hyperacetylation of histones H4 and H2A. **A.** Enhanced reactivity of KM-2, anti-H4-AcK5, anti-H4-AcK8, anti-H4-AcK12, anti-H4-AcK16, anti-H2A-AcK5, anti-H2A-AcK9 and anti-H2B-PhS14 with histones isolated from CPT-induced (for 4h) apoptotic Jurkat cells. An antibody specific for actin was used as an internal loading control. (continues next page)

B. KM-2 recognizes apoptotic blebs of campthothecin-induced apoptotic Jurkat cells that positively stain by TUNEL (see arrows). DAPI staining reveals defragmentation of the nucleus. **C. and D.** Enhanced reactivity of KM-2 with histones H4 and H2A, extracted from CPT- and anti-Fas-induced apoptotic Jurkat cells coincides with cleavage of caspase-3. The progress of apoptosis was analyzed by AnnexinV and Propidium Iodide (PI) staining.

Also anti-FAS-induced apoptotic cells were analyzed to exclude a solely CPT-specific effect. Already 1 hour after CPT or 4 hours after anti-FAS treatment, the reactivity of KM-2 with histone H4 and histone H2A increased with a concomitant cleavage of caspase-3 (Fig. 2, C and D). Notably, one hour after induction of apoptosis with CPT the percentage of apoptotic cells was still extremely low as measured by AnnexinV-FITC staining, which indicates that apoptosis-induced acetylation of histone H4 and histone H2A is a very early event that precedes the flip-flop of phosphatidyl serine in the lipid bi-layer (Fig. 2, C and D). The enhanced reactivity of KM-2 with apoptotic histone H4 and histone H2A was not Jurkat-specific, since similar results were obtained with the monocytic cell line U937 (unpublished data).

We then tested whether apoptosis-induced changes in expression and/or activity of histone acetyltransferases (HATs) or histone deacetylases (HDACs) could serve as a mechanistic explanation for the observed hyperacetylation of apoptotic histones. Indeed, in Jurkat cells an increased expression of the HAT protein p300 could be demonstrated already 1 hour after induction of apoptosis with CPT, while the expression of the two HAT proteins CBP and pCAF, and the HDAC enzyme HDAC1 did not change upon induction of apoptosis (Fig. 3A). In addition to protein expression we also determined total HAT and HDAC activity in both normal and apoptotic Jurkat cells. It appeared that total HDAC activity was significantly lower 4 hours after induction of apoptosis in either CPT- or anti-FAS induced apoptotic Jurkat cells, while total HAT activity was significantly increased in the CPT-treated cells, but not in the anti-FAS-treated apoptotic Jurkat cells (Fig. 3B). Taken together, the apoptosis-induced hyperacetylation of histone H4 and histone H2A, which is recognized by the lupus-derived autoantibody KM-2, is most likely mediated by an increased HAT activity and/or a decreased HDAC activity.

To extend the findings obtained with the lupus-derived monoclonal autoantibody KM-2, the reactivity of plasmas derived from 6-9-weeks-old (pre-diseased) and 20-weeks-old (diseased) MRL/lpr lupus mice with non-acetylated and acetylated histone H4 peptides (residues 1-22) was tested in ELISA and expressed as a ratio (acetylated/non-acetylated). Plasmas from both pre-diseased and diseased lupus mice showed a significantly higher reactivity with histone H4 peptides acetylated at K8, K12 or K16 compared to the non-acetylated histone H4 peptide, i.e. the ratio acetylated/non-acetylated was significantly higher than 1, while the reactivity with histone H4 peptide tetra-acetylated at K5, K8, K12 and K16 did not

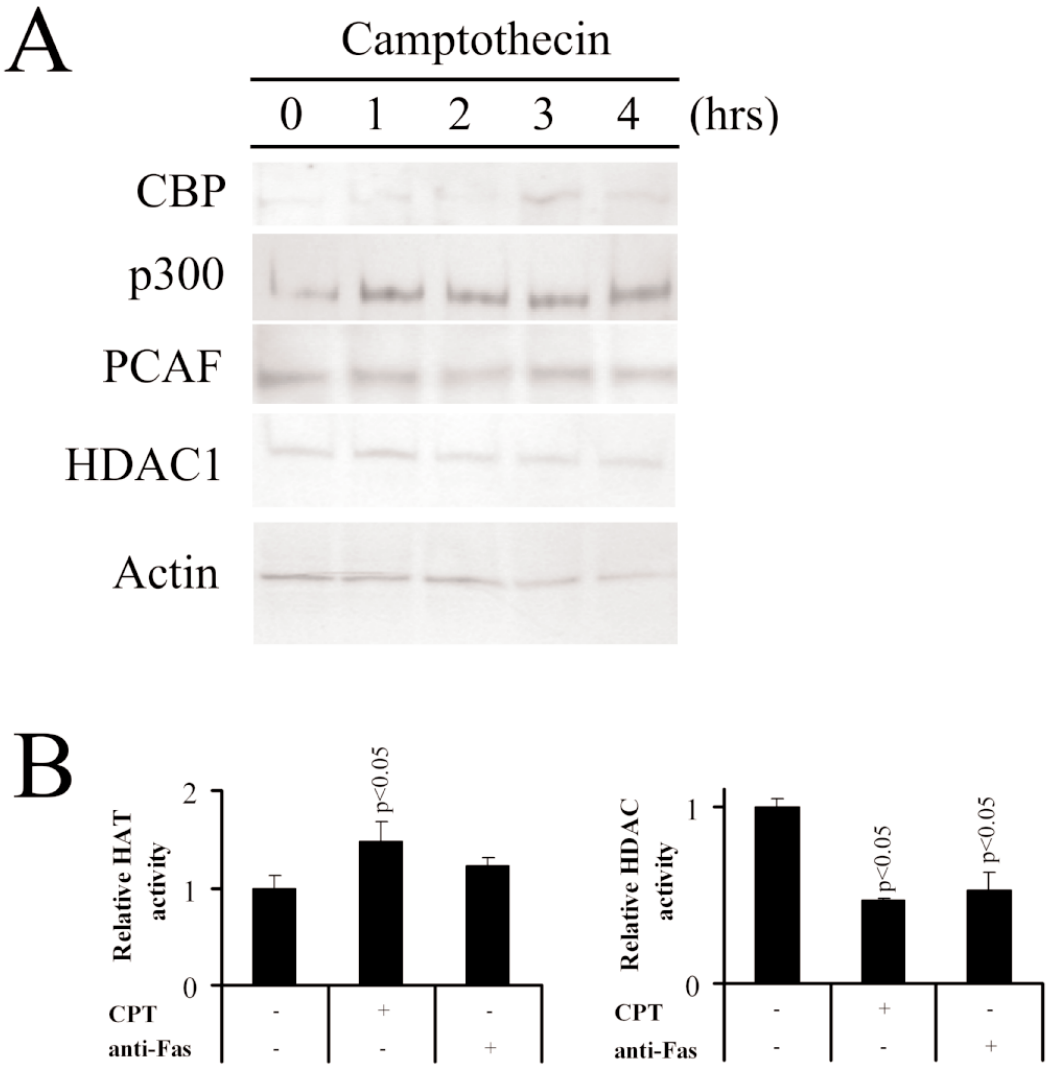

Figure 3. Expression and activity of histone acetyltransferases and histone deacetylases in non-apoptotic and early apoptotic cells. **A.** Protein expression analysis of histone acetyltransferases CBP, p300 and P/CAF, and histone deacetylase 1 (HDAC1) in non-apoptotic and campthothecin or anti-Fas-induced early apoptotic Jurkat cells. An antibody specific for actin was used as an internal loading control. **B.** HAT and HDAC activity in non-apoptotic and campthothecin or anti-Fas-induced early apoptotic Jurkat cells. Significant differences are indicated.

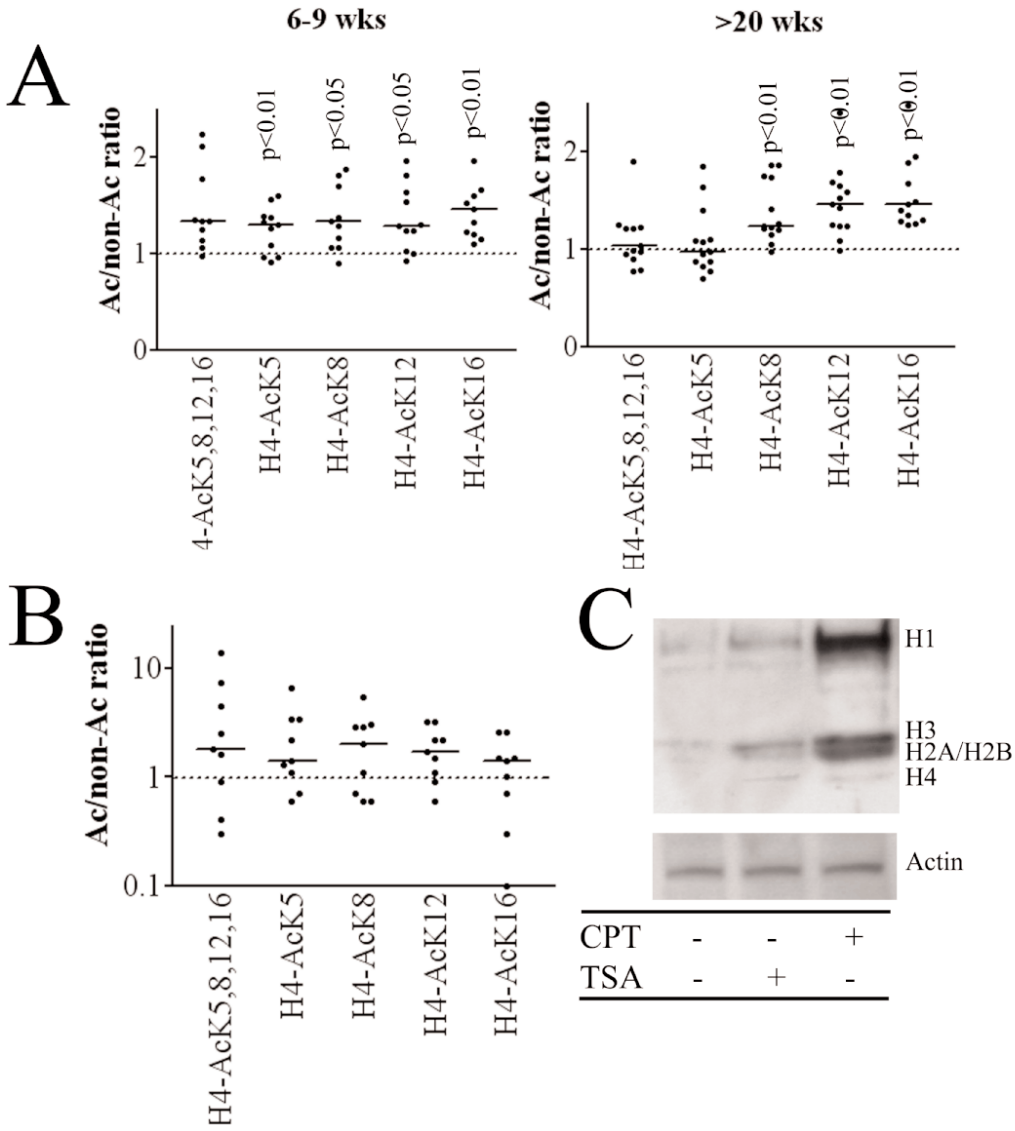

Figure 4. Lupus-derived plasmas recognize acetylated and apoptotic histones. **A.** Reactivity of plasmas from pre-diseased (left panel) and diseased (right panel) MRL/lpr lupus mice with (acetylated) histone H4 peptides (residues 1-22) [$p<0.01$, $p<0.05$ compared to non-acetylated]. **B.** Reactivity of plasmas from SLE patients with (acetylated) H4 peptides (residues 1-22). **C.** Enhanced reactivity of lupus-derived plasmas with histones extracted from both CPT- and TSA-treated Jurkat cells

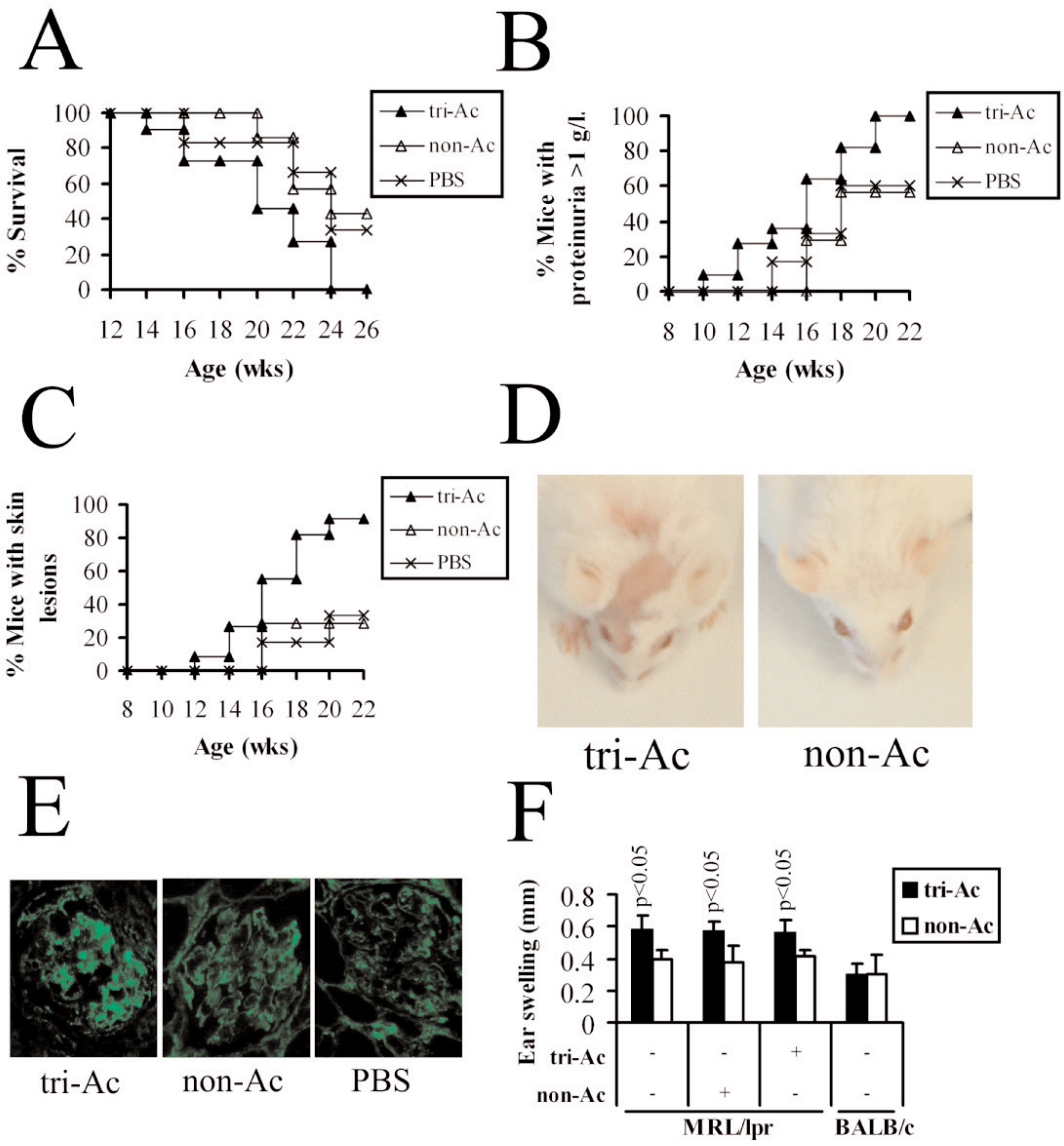

Figure 5. Tri-acetylated histone H4 peptide accelerates disease in MRL/lpr lupus mice. Treatment of 8-weeks-old MRL/lpr lupus mice with either non-acetylated H4 peptide (residues 1-22; n=7), H4 peptide tri-acetylated at lysine residues 8, 12 and 16 (n=11) or PBS as a control (n=6). **A.** Tri-acetylated H4 peptide results in enhanced mortality ($p<0.01$). **B.** Tri-acetylated H4 peptide aggravates proteinuria ($p<0.05$). **C. and D.** Tri-acetylated H4 peptide induces early and severe skin lesions ($p<0.05$). **E.** Tri-acetylated H4 peptide enhances glomerular IgG depositions (score 2.1±0.2) compared to non-acetylated H4 peptide (score 1.3±0.1) and PBS (score 0.8±0.2) treatment ($p<0.01$). **F.** DTH response of 16-18-weeks-old MRL/lpr mice against tri-acetylated H4 peptide is higher than against non-acetylated H4 peptide in all groups ($p<0.05$ in all groups). BALB/c mice did not show a significant DTH response against the H4 peptides. PBS injections gave an average ear swelling of 0.2 mm.

significantly differ (Fig. 4A, left and right panel). Plasmas from diseased lupus mice showed reactivity with histone H4 peptide acetylated at K5 similar to the non-acetylated histone H4 peptide (Fig. 4A, right panel). Therefore, acetylation of histone H4 at lysine residues 8, 12 and 16 seems to be important. We also tested plasmas from 6-9-weeks-old (pre-diseased) and 30-weeks-old (diseased) (NZWxNZB)F1 lupus mice, that have a different MHC haplotype compared to MRL/lpr lupus mice, but which yielded comparable results (unpublished data). The reactivity of plasmas from several SLE patients with (acetylated) H4 peptides revealed on average a 1.5-2-fold enhanced reactivity for all acetylated peptides, but due to a large variance the observed differences were not statistically significant (Fig. 4B). Notably, plasmas from MRL/+ control mice and healthy individuals did not show any reactivity in ELISA with the histone H4 peptides, either acetylated or non-acetylated (unpublished data). When acid-extracted histones from normal and apoptotic Jurkat cells were used as a coating in ELISA, a 2-fold higher reactivity of plasmas from both lupus patients and mice with apoptotic histones compared to non-apoptotic histones could be demonstrated (unpublished data). Most likely, this higher reactivity of lupus-derived plasmas with apoptotic histones compared to normal histones may not only be explained by apoptosis-induced hyperacetylation of histones but also by other apoptosis-induced histone modifications. Nevertheless, probing of blots with plasmas from SLE patients revealed an enhanced reactivity of the vast majority of plasmas with equally loaded and resolved histones H4, H2A, H2B, H3 (and most likely H1) extracted from both TSA- and CPT-treated Jurkat cells (Fig. 4C). The superior reactivity with histones derived from CPT-treated cells compared to histones derived from TSA-treated cells may be explained by other apoptosis-induced histone modifications. Taken together, lupus-derived plasmas do recognize apoptosis-induced histone modifications that include histone acetylation.

Tri-acetylated H4 peptide accelerates disease development in lupus mice

To evaluate whether apoptosis-induced histone acetylation is important for the pathogenesis of SLE, non-acetylated histone H4 peptide (residues 1-22) and tri-acetylated (at K8, K12 and K16) histone H4 peptide were subcutaneously administered to BALB/c and pre-diseased MRL/lpr lupus-prone mice, while PBS-injected mice were included as a control. Administration of histone H4 peptide, either acetylated or non-acetylated, to BALB/c mice did not result in autoimmunity and proteinuria (unpublished data). However, administration of the tri-acetylated histone H4 peptide to pre-diseased MRL/lpr lupus-prone mice resulted in an enhanced mortality compared to treatment with the non-acetylated histone H4

peptide or saline (Fig. 5A). Furthermore, the development of proteinuria and progression of skin lesions was significantly accelerated and affected more mice in the group treated with the tri-acetylated histone H4 peptide compared to the saline-treated or non-acetylated histone H4 peptide-treated groups (Fig. 5, B and C). In addition, the severity of skin lesions was more pronounced in the group that received tri-acetylated histone H4 peptide (Fig. 5D). The titers of the anti-dsDNA, anti-histone and anti-nucleosome antibodies were not significantly different between the different groups of mice as determined in ELISA (unpublished data). In lupus glomerular IgG deposition is associated with the development and the severity of proteinuria. Therefore, we analyzed frozen kidney sections from diseased mice for the presence of IgG, and scoring on a scale of 0-3 revealed a glomerular IgG-deposition index of 2.1 (± 0.2) for the group treated with the tri-acetylated histone H4 peptide, which was significantly ($p< 0.01$) higher compared to 1.3 (± 0.1) and 0.8 (± 0.2) for the groups treated with the non-acetylated histone H4 peptide or PBS, respectively (Fig. 5E). Next, we wanted to test whether the acceleration of disease development by the tri-acetylated histone H4 peptide was associated with specific T cell activity. Unfortunately, a mixed lymphocyte reaction (MLR) that included syngeneic antigen presenting cells loaded with (acetylated) histone H4 peptides and responder cells isolated from spleen and lymph nodes did not yield reliable data (unpublished data). Therefore, we applied the delayed type hypersensitivity reaction (DTH). Mice (16-18-weeks-old) pre-treated with the non-acetylated histone H4 peptide, the tri-acetylated histone H4 peptide or PBS, received either non-acetylated histone H4 peptide or tri-acetylated histone H4 peptide in the left or right ear, respectively, and subsequently ear swelling was measured after 24 and 48 hours. In all groups ear swelling induced by the tri-acetylated histone H4 peptide was significantly greater than the ear swelling induced by the non-acetylated histone H4 peptide (Fig. 5F). Apparently, mice that were pre-treated with only PBS showed already a higher DTH response against the tri-acetylated histone H4 peptide than against the non-acetylated histone H4 peptide. Thus, pre-treatment with tri-acetylated histone H4 peptide did not further increase the DTH response against tri-acetylated histone H4 peptide. As a control we determined the DTH against the tri-acetylated and non-acetylated histone H4 peptide in BALB/c mice, which did not reveal a significant different response against either peptide (Fig. 5F). Taken these data together, administration of tri-acetylated histone H4 peptide, which corresponds to the apoptosis-induced epitope for the lupus-derived autoantibody KM-2, significantly accelerates disease development in MRL/lpr lupus-prone mice.

Discussion

We show for the first time that a SLE-derived monoclonal autoantibody (KM-2) shows an enhanced reactivity with hyperacetylated histone H4 and histone H2A derived from either apoptotic cells or cells treated with a histone deacetylase inhibitor. Most importantly, the primary epitope for KM-2 seems to be generated by apoptosis. These observations were not limited to this specific autoantibody but were also found with plasmas from both lupus patients and mice. Other groups also have reported that lupus-derived sera show (enhanced) reactivity with apoptotic histones, however, the nature of these histones was not evaluated (175).

We demonstrate that apoptosis-induced acetylation of histones was mediated by changes in HDAC and/or HAT expression and/or activity. Both apoptosis-induced histone deacetylation and acetylation have been described, which in some cases was associated with changes in the HDAC or HAT activity and/or expression (25-28, 176, 177). These apparently contradictory results may be explained by differences in applied experimental approaches, i.e. differences in cell types, methods of apoptosis induction and extraction of histones, and subsequent analysis of histone acetylation. In this study we have characterized and applied a sensitive tool that was provided by nature itself, i.e. a lupus-derived monoclonal autoantibody, to analyze apoptosis-induced histone acetylation. In agreement with our results, cis-platin induced apoptosis results in global hyperacetylation of histones (25). In addition, treatment by HDAC inhibitors like TSA, which results in global hyperacetylation of histones, also leads to apoptosis, while HDAC inhibitors and other inducers of apoptosis seem to act synergistically in apoptosis induction (177, 293). However, the apoptosis-inducing capacity of HDAC inhibitors may be explained by the effect of the induced histone hyperacetylation on the expression of genes operative in apoptotic pathways or the cell cycle (294, 295), since in general hyperacetylation of histones leads to a transcriptionally active and open chromatin structure (296). Other apoptosis-induced histone modifications that have been described include de-ubiquitination of H2A, transglutamination of H2B, and phosphorylation of H2A, H2A.X, H2B and H3 at different residues (24). Apoptosis-induced phosphorylated proteins have been demonstrated to be targets for SLE-derived sera, however phosphorylated histones were not evaluated (143). As far as we know apoptosis-induced methylation (or demethylation) or citrullination of histones has not been assessed yet. This latter modification is of particular importance, since antibodies specific for citrullinated autoantigens are predictive for the development of rheumatoid arthritis (149). Nevertheless, until now none of the mentioned apoptosis-induced histone modifications have been identified as targets of autoantibodies in SLE.

Several nucleosomal and/or histone T cell epitopes have been identified for both lupus mice and patients in H2A, H2B, H3 and H4 (55-58, 178, 227). However, in all these studies the T cell response was measured by presenting a panel of unmodified histone peptides, which could have prevented the identification of T cell epitopes containing modifications. Apoptosis-associated histone modifications that may be located within the identified lupus T cell epitopes include de-ubiquitinaton of histone H2A at position 119, phosphorylation of histone H2B at S14, and acetylation of histone H4 at K16 (this work;18;179). It has been shown that administration of apoptotic cells or apoptotic cells in combination with either dendritic cells or incomplete Freund's adjuvant, results in the development of anti-nucleosome antibodies and proteinuria in both normal and lupus mice (28, 171, 173). Here, we show that administration of a histone H4 peptide tri-acetylated at K8, K12 and K16 accelerates disease development in pre-diseased lupus-prone mice, as measured by the survival rate, the presence of glomerular IgG depositions, and the development of proteinuria and skin lesions. In contrast to lupus-prone mice, BALB/c mice treated with the tri-acetylated histone H4 peptide failed to develop autoimmunity, which may be explained by the lack of genetic predisposition. Importantly, the DTH response in MRL/lpr mice against the tri-acetylated histone H4 peptide was higher than against the non-acetylated histone H4 peptide and was independent from the pre-treatment, i.e. saline, non-acetylated histone H4 or tri-acetylated histone H4 peptide. Moreover, BALB/c mice did not show a DTH response against either the tri-acetylated or non-acetylated histone H4 peptide. These findings strongly suggest that the tri-acetylated histone H4 peptide that caries the apoptosis-associated acetylation motif incites a more vigorous immune response than the corresponding non-acetylated histone H4 peptide, which depends on the genetic lupus background. It remains to be established whether in addition to apoptosis-induced histone acetylation other apoptosis-induced histone modifications (or combinations of histone modifications) exist, i.e. a "histone code" for apoptosis, which are targets of lupus-derived autoantibodies and pathogenic (14, 291). In this context it would be of considerable interest to determine in patients whether antibodies specific for apoptosis-induced histone or nucleosome modifications are predictive for the development of SLE and/or exacerbations of the disease. Furthermore, the apoptosis-induced histone motifs that are targets for autoantibodies in lupus could be applied in tolerizing strategies (297).

In conclusion, we have shown that lupus-derived autoantibodies recognize apoptosis-induced histone modifications, in particular histone acetylation, and that a histone H4 peptide carrying an apoptosis-induced acetylation motif accelerated disease development and severity in pre-diseased lupus-prone mice. Therefore, the challenge of the immune system by apoptosis-induced chromatin modifications may

be an important initial event that leads to the disruption of the balance between tolerance and autoimmunity and the formation of pathogenic chromatin autoantibodies in SLE.

Acknowledgements

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Chapter 4.

Decreased phagocytosis of apoptotic cells in diseased lupus mice

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Abstract

Antibodies against nucleosomes are a serological hallmark of systemic lupus erythematosus (SLE). Apoptotic cells are the unique source of nucleosomes, which are formed through cleavage of chromatin by nucleases. These nucleosomes and other autoantigens targeted in SLE are expressed in apoptotic blebs or at the surface of apoptotic cells. Therefore, it is conceivable that circulating antibodies can influence apoptotic cell clearance. Using an *in vitro* phagocytosis assay, we analysed the phagocytic efficacy for apoptotic cells of resident peritoneal macrophages from pre-morbid and diseased lupus mice. The assay was carried out in the presence of autologous serum, using autologous apoptotic thymocytes as targets. Under these conditions macrophages from diseased MRL/lpr and NZBxNZW(F1) lupus mice, and from age-matched NZB mice showed a decreased phagocytic efficacy (decrease 47%, 48% and 37%, respectively compared to measurements in pre-morbid mice). The cause of this decrease resides in the serum, and is not due to an acquired defect of macrophages. In conclusion, during disease progression in murine SLE, apoptotic cell clearance becomes impaired, which might amplify further disease progression.

Normally, apoptotic cells are swiftly removed by phagocytosis due to surface changes induced by the apoptotic process (40, 121). This prevents the release of phlogistic intracellular constituents, including nucleosomes, which are uniquely formed during apoptosis through cleavage of chromatin by nucleases. However, antibodies against nucleosomes are a hallmark of systemic lupus erythematosus (SLE) (13). Therefore, disturbances in either apoptosis or the phagocytosis of apoptotic cells have been proposed to play a role in the development of autoimmunity, especially in SLE (11, 13, 102, 121, 173, 180, 181). Several reports using mice deficient for molecules (such as C1q, SAP and IgM) or receptors involved in the phagocytosis of apoptotic cells (truncated Mer receptor) have shown that a disturbed removal of apoptotic cells leads to autoimmunity against nucleosomes and glomerulonephritis (40). Furthermore, defects in the clearance of apoptotic cells have been described in lupus mice and patients (43, 44, 46, 118). Nucleosomes are not only important for disease induction, they can also influence the clearance of apoptotic cells (182). Furthermore, autoantibodies formed in SLE can also modulate this process (183, 184). Therefore, during disease progression, when nucleosomes and autoantibodies start to circulate, they might amplify disease by inhibiting clearance of apoptotic cells.

To test whether phagocytic efficacy is disturbed in murine lupus, we developed an *in vitro* phagocytosis assay of apoptotic thymocytes to determine the efficacy of resident peritoneal macrophages. During development of the assay we

found that the uptake of apoptotic cells by mouse resident peritoneal macrophages was fully dependent on serum (142). This allowed the evaluation of the impact of autologous serum of pre-morbid and diseased SLE-mice on the phagocytic efficacy. To mimic the *in vivo* situation measurements were performed in an autologous system, using autologous apoptotic thymocytes as target cells and autologous resident peritoneal macrophages as effector cells.

Materials & Methods

Animals

For all experiments, female mice were used. Young, pre-morbid mice were 6–12 weeks of age. Diseased MRL/lpr mice were 20–24 weeks old. Age-matched MRL/+ mice were also included in the study. Diseased NZBxNZW(F1) mice were either 15 weeks or 30–34 weeks old (full blown disease), NZW and NZB mice of 30–34 weeks were also tested. Lupus disease was defined by the presence of albuminuria of >0.3 g/l as determined by Albustix (Roche, Mannheim, Germany) and an anti-DNA titer >5000 U/ml in ELISA determined as described previously (51, 94). The animals were housed under specific pathogen-free conditions and were fed standard food (Hope Farms, Woerden, The Netherlands) and acidified tap water ad libitum. NZ mice were purchased from Harlan/Olac (Bicester, UK), MRL mice from Bomholdgård (Ry, Denmark) and BALB/c, Swiss and CBA mice were purchased from Harlan (Horst, The Netherlands).

Medium

RPMI Dutch modification (Gibco, Paisley, Scotland) was used as medium, supplemented with 1 mM sodium pyruvate (ICN, Aurora, Ohio), 0.3 mg/ml L-glutamine (Flow Laboratories, Zwanenburg, The Netherlands), 100 IU penicillin (Gibco, Paisley, Scotland, UK), 100 µg/ml streptomycin. This solution is further referred to as serum-free medium. Further supplements to serum-free medium were either fetal calf serum (FCS) (Gibco, Paisley, Scotland, UK), heat inactivated for 30 min at 56°C, mouse serum (MS), not heat inactivated, or human serum albumin (HSA) (CLB, Amsterdam, The Netherlands). These solutions are further referred to as x% FCS, x% MS and x% HSA, respectively. Mouse serum was obtained by bleeding from the retro-orbital plexus after ether anaesthesia. Serum was collected in glass tubes, allowed to clot for 30 min at room temperature and 30 min at 4°C, and centrifuged at 2300 x g, after which the supernatant was harvested. This supernatant was again centrifuged at 2300 x g and its respective supernatant harvested. The twice-spun serum was used immediately.

Isolation of thymocytes and induction of apoptosis

Thymocyte isolation and apoptosis induction was performed as described (142). In short, from the thymus a single-cell suspension of thymocytes was prepared, which was seeded at $5x10^6$ cells/ml in 10% FCS and exposed to 1 µM

dexamethasone (Genfarma, Maarssen, The Netherlands) at 37°C, 5% CO_2. After 3 h, the cells were washed three times in serum-free medium and brought to a concentration of $2.5x10^6$/ml in either an autologous serum solution or in a FCS solution. Dexamethasone induced apoptosis was analysed by annexin V-FITC/propidium iodide double staining in a Coulter Epics XL flowcytometer (Coulter Corporation, Hialeah, FL, USA).

Isolation of resident peritoneal macrophages

This isolation was performed as described (142). In short, non-anaesthetised mice were injected with 10 ml ice-cold 10% FCS, followed by gravity drainage through a 1.1 mm Ø needle. A sample was taken for forward scatter/sideward scatter (FS/SS) analysis on a Coulter Epics XL flowcytometer (Coulter Corporation, Hialeah, FL, USA) to determine the percentage of macrophages in the lavage. Peritoneal macrophages were seeded on plastic coverslips at $7.5x10^4$ cells/ml in 10% FCS and allowed to adhere for 1.5 h at 37°C, 5% CO_2, after which non-adherent cells were removed by washing with 10% FCS. The coverslips were transferred to new wells containing 1 ml of 10% FCS and the macrophages were kept at 37°C, 5% CO_2 for an additional 1.5 h, during which time the apoptic thymocytes were prepared.

Macrophage and apoptotic cell interaction

Plastic coverslips with adherent macrophages were washed with serum-free medium after which 1 ml of dexamethasone treated thymocytes ($2.5x10^6$/ml) in medium containing the appropriate serum (either FCS or MS) was gently added to each well. The cells were allowed to interact for 1 h at 37°C, 5% CO_2, after which the coverslips were washed with 0.4% HSA to remove non-phagocytosed cells. For all autologous experiments, macrophages, serum and thymocytes were obtained from the same mouse.

Scoring procedure

Scoring was performed as described (142). In short, resident peritoneal macrophages were flattened and dried by centrifugation of the coverslips at 25 x g for 10 min in a Hettich Universal 30F table centrifuge (Depex, De Bilt, The Netherlands). Cells were fixed with methanol 99.9% (LabScan, Dublin, Ireland), and stained with May–Grünwald/Giemsa (Merck, Darmstadt, Germany). Preparations were scored at 400x magnification by regular light microscopy and the number of apoptotic thymocytes per individual macrophage was determined for 100

Mϕs for each coverslip. Only thymocytes clearly within the perimeter of the macrophage were counted.

Statistical analysis

All tests were performed in duplicate or triplicate, and for each experiment the counts of 200 or 300 Mϕs, respectively, were used. The resulting mean is expressed as "number of thymocytes per macrophage". The results from two or more experiments are given as mean±SD. Student's t-test and one-way ANOVA in combination with Bonferroni's post-test correction for multiple comparisons were applied when appropriate.

Results

Titration of autologous serum concentration

The initial assay was developed with fetal calf serum (FCS) (142). In order to determine the optimal concentration for mouse serum (MS), we compared the phagocytic efficacy in FCS and MS. Fig. 1 shows the uptake of apoptotic BALB/c thymocytes by BALB/c resident peritoneal macrophages in the presence of different concentrations of BALB/c serum, compared to the uptake in the presence of FCS. Autologous BALB/c serum is less efficient compared to fetal calf serum at concentrations of 2.5% or lower. Therefore, a MS concentration of 5% was chosen for further experiments. A higher concentration was not feasible from a practical point of view.

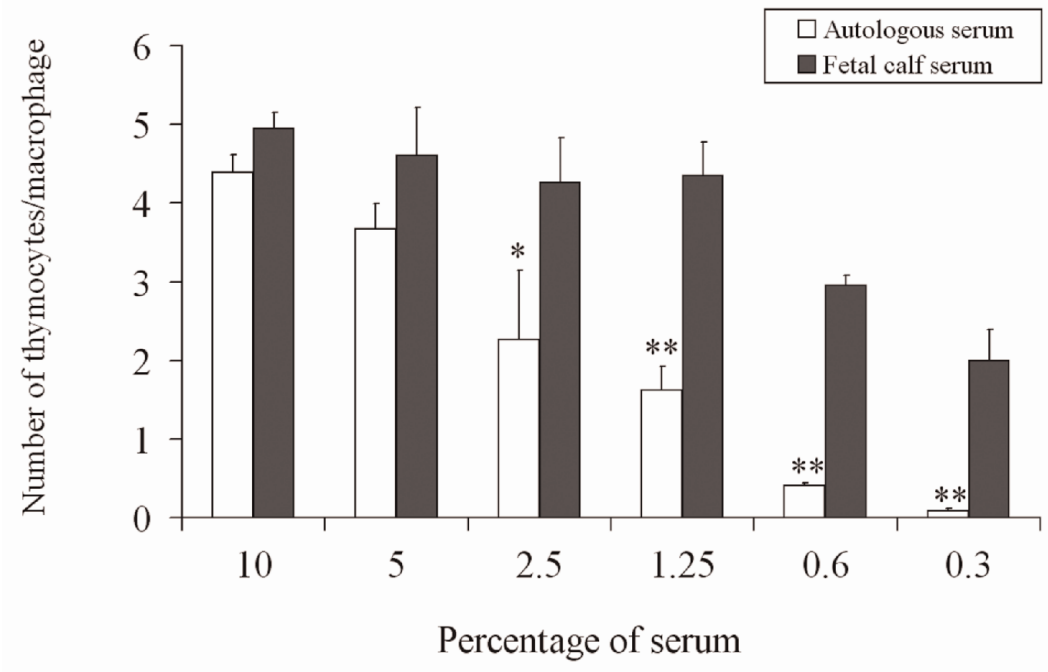

Figure 1. Effect of the concentration of autologous mouse serum or fetal calf serum on the efficacy of phagocytosis of apoptotic cells. BALB/c resident peritoneal macrophages were allowed to phagocytose apoptotic BALB/c thymocytes in autologous BALB/c serum or heterologous fetal calf serum. Results are expressed as the number of thymocytes per macrophage and are the mean±SD of at least two measurements per concentration (*P<0.05, **P<0.001).

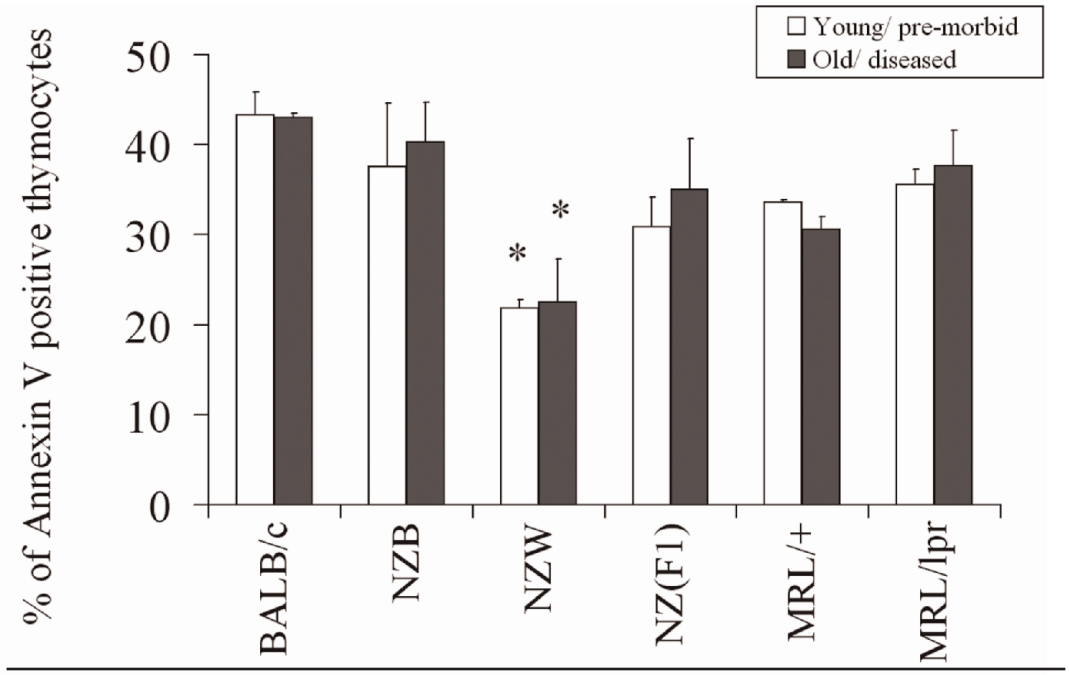

Figure 2. Percentage of dexamethasone induced annexin V positive thymocytes in the different mouse strains. Results are expressed as the percentage of annexin V positive thymocytes and are the mean±SD of at least four measurements per strain. Although there were strain differences in the magnitude of apoptosis induction, there were no differences within a given strain for mice of 6–12 weeks and mice of 20–24 weeks (MRL) or 30–34 weeks (NZ, BALB/c) (*P<0.001).

Apoptosis induction in autologous thymocytes

To test if apoptosis induction was comparable in thymocytes from mice of 6–12 weeks and from mice >20 weeks, thymocytes were treated for 3 h with dexamethasone. Apoptosis induction was measured by annexin V binding. There were no differences in the percentage of annexin V positive cells from the two groups within a given strain, although there were marked differences between the strains with respect to the sensitivity for dexamethasone induced apoptosis. This sensitivity was highest in BALB/c thymocytes, and lowest in thymocytes from NZW mice (Fig. 2).

Phagocytic efficacy under autologous conditions

The phagocytic efficacy of resident peritoneal macrophages from mice of 6–12 weeks and >20 weeks of lupus-prone strains and control strains under fully autologous conditions is shown in Fig. 3. When disease becomes apparent, the

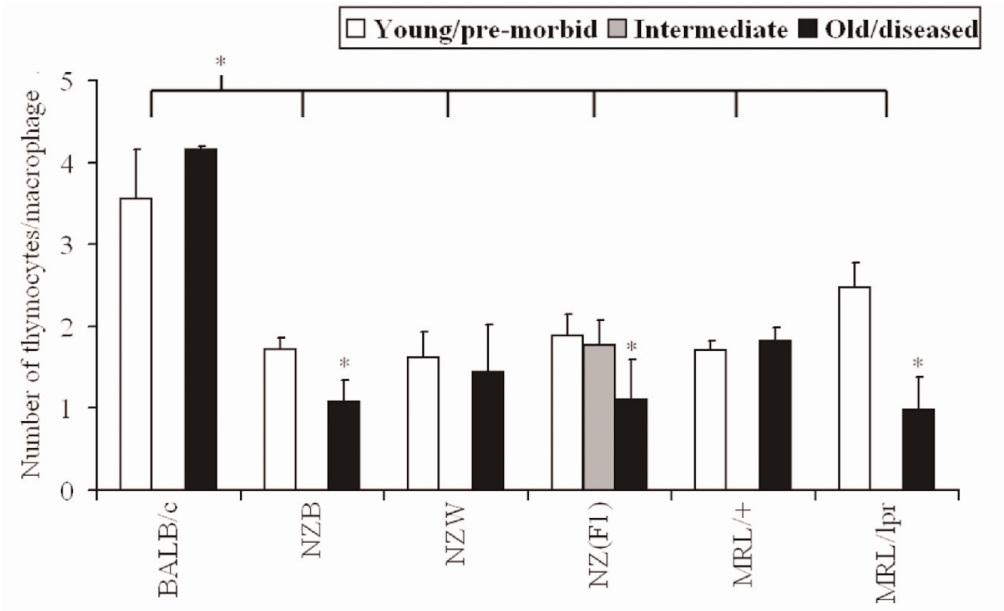

Figure 3. Phagocytic efficacy of resident peritoneal macrophages from mice under autologous conditions. Mice were 6–12 weeks (pre-morbid), 25 weeks (early disease; NZ(F1)), 20–24 weeks (diseased; MRLlpr and MRL/+) or 30–34 weeks (diseased; NZ(F1), or control NZB, NZW and BALB/c). Resident peritoneal macrophages were allowed to take up apoptotic autologous thymocytes in 5% autologous serum for 1 h. Results are expressed as the number of thymocytes per macrophage and are the mean±SD of at least four measurements per strain (*P<0.05).

efficacy of apoptotic cell phagocytosis becomes compromised in the SLE strains NZBxNZW(F1) and MRL/lpr. In mice aged 30–34 weeks of the SLE-parental strain NZB, phagocytic efficacy is also reduced in comparison to results obtained in NZB mice of 6–12 weeks. When comparing different strains at 6–12 weeks, efficacy in BALB/c is higher compared to efficacies from all other strains ($P<0.05$). Phagocytosis efficacy in MRL/lpr of 6–12 weeks was not statistically different from that in MRL/+ mice of the same age. To determine at which age the reduced efficacy of phagocytosis would become apparent, the efficacy of albuminuria negative, but anti-DNA positive, NZBxNZW(F1) mice of 25 weeks of age were tested. At 25 weeks of age, the efficacy was slightly reduced, but this difference was not significant compared to NZWxNZBF1 mice of 6–12 weeks (Fig. 3).

Phagocytic efficacy under heterologous conditions

To determine which component in the autologous system causes the reduction in phagocytic capacity, resident peritoneal macrophages from 30–34

weeks old, diseased NZBxNZW(F1) mice were exposed to their autologous apoptotic thymocytes in the presence of 5% FCS (Fig. 4, bar 3). The differences measured under fully autologous conditions now were no longer apparent. Moreover, serum from 30–34 weeks old, diseased NZBxNZW(F1) mice caused a decrease in the phagocytic capacity of macrophages from premorbid (6–12 weeks) NZBxNZW(F1) mice (Fig. 4, bar 4). Furthermore, in both the MRL/lpr and the NZBxNZW(F1) model there was no difference in phagocytosis by macrophages from pre-morbid or diseased mice when the assay was performed with apoptotic thymocytes from BALB/c as target cells and 10% FCS as source of serum (data not shown). These findings suggest that there is no age-related or disease-related decrease of macrophage function, and that the cause of the decrease in efficacy resides in the serum.

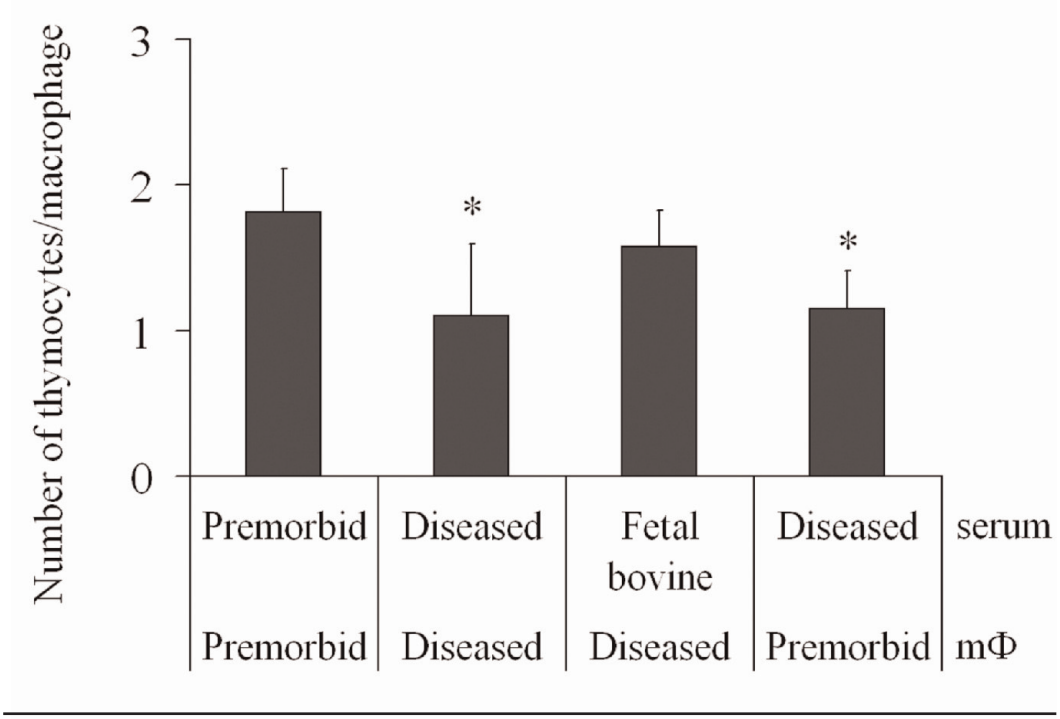

Figure 4. Phagocytic efficacy under autologous versus heterologous conditions of resident peritoneal macrophages (NZWxNZB)F1 mice using autologous apoptotic thymocytes as targets. Results are expressed as the number of thymocytes per macrophage and are the mean±SD of four measurements per condition (*P<0.05). Bar 1: efficacy of macrophages of 6–12 weeks old premorbid mice in the presence of serum of the same mice (premorbid serum). Bar 2: efficacy of macrophages of 30–34 weeks old diseased mice in the presence of serum of the same mice (diseased serum). Bar 3: efficacy of macrophages of 30–34 weeks old diseased mice in the presence of fetal calf serum. Bar 4: efficacy of macrophages of 6–12 weeks old premorbid mice in the presence of serum of 30–34 weeks old mice (diseased serum).

Discussion

This study shows that with disease progression the efficacy of peritoneal macrophages to ingest apoptotic cells decreases in lupus mice. This finding is in line with recent evidence that in lupus patients the phagocytosis of apoptotic cells is impaired (43, 44, 46, 118). The described effect is likely to promote the release of nucleosomes in the circulation, which in turn might enhance disease progression.

When the autologous serum from premorbid SLE mice was replaced by serum from diseased SLE mice, the phagocytosis of apoptotic cells decreased. In addition, when the autologous serum from diseased SLE mice was replaced by FCS, the decrease in phagocytic capacity was no longer observed. Furthermore, peritoneal macrophages from diseased mice showed a normal phagocytic capacity when apoptotic thymocytes from BALB/c mice were used as targets in the presence of normal FCS. The decrease in phagocytic capacity was not seen in control mice, except for the lupus-parental strain NZB. Therefore, the decreased phagocytosis of apoptotic cells can not only be ascribed to an inherent defect of macrophages from older lupus mice. This has been suggested in reports in SLE on decreased complement-receptor (185) and Fc-receptor (186) mediated phagocytosis and the Mer receptor deficient mouse (133), which suggested that SLE related phagocytic defects are present at the macrophage level. At first sight, this contrasts with previous reports (43, 44, 46, 118), especially with the observation of Potter et al., which showed a decrease in phagocytosis of apoptotic cells in premorbid (age 8–12 weeks) lupus-prone mice compared to the control strain BALB/c and C57Bl/6. However, we also observed a lower phagocytic clearance of apoptotic cells using 10% FCS, in lupus strains compared to BALB/c both at 6–12 weeks and >20 weeks. This is possibly related to the use of BALB/c mice as control, since we found a similar, lower phagocytic clearance of apoptotic cells in normal CBA mice, which is a H-2 haplotype identical strain for MRL mice (42). We did not test C57Bl/6 mice in this study. This suggests that the difference in phagocytic efficacy of premorbid lupus mice, as observed by others (43, 44, 46, 118), is due to the intrinsic higher efficacy in some (BALB/c) but not all (CBA) normal strains.

An apoptosis induced decrease of the number of macrophages ingesting apoptotic cells has been put forward as a possible explanation for the decrease in phagocytic clearance (43, 44, 46, 118). In contrast to these reports, we did not find a decrease in the percentage of macrophages ingesting apoptotic cells. We observed a shift of the distribution curve of apoptotic cells per macrophage towards zero (data not shown). We also found no decrease in the recovery of macrophages from the peritoneal cavity for lupus strains compared to control strains. In a previous study in pre-morbid lupus mice, we also found no evidence for a constitutive defect in the phagocytosis of apoptotic cells (42).

The observed decrease in apoptotic cell phagocytosis is more likely related to one or more factors residing in the serum. This could be due to an increase of some inhibitory factor(s), or alternatively result from a decreased concentration of a serum factor required for phagocytosis. The decrease could be due to either a defective binding or internalisation of apoptotic cells by macrophages. In a previous study, we could show that uptake and binding are different processes (142). However, only the uptake of apoptotic cells by macrophages is critically dependent of serum. Therefore, we can conclude that the internalisation becomes impaired. There are several putative candidates whose increased presence could lead to a decrease in apoptotic cell phagocytosis. First, nucleosomes have been shown to be able to inhibit phagocytosis of apoptotic cells (182) This would be in line with our recent data showing that diseased SLE mice indeed have nucleosomes in their circulation (51). However, there appears to be no correlation between the amount of nucleosomes in the serum and the phagocytic capacity. We found that the amount of nucleosomes is high in the NZB and MRL/+ background strains, and lower in the NZBxNZW(F1) and MRL/lpr strains (51). These two latter strains show decreased phagocytic efficacies, while MRL/+ mice do not. Moreover, young mice from both SLE and control strains have nucleosomes in their circulation (51), but they do not have a decreased phagocytic capacity. Therefore, circulating nucleosomes are probably not responsible for the observed impairment of phagocytosis of apoptotic cells in NZB, NZBxNZW(F1) and MRL/lpr mice.

A second candidate responsible for the observed decrease in phagocytosis could be the presence of autoantibodies against several structures expressed on apoptotic cells like the β2-glycoprotein-1/phophatidylserine complex. Indeed, antibodies to phosphatidyl-serine have been implicated in the modulation of apoptotic cell clearance (183, 184). However, to our knowledge autoantibodies against phosphatidylserine have not been reported in NZB mice, yet serum from these mice also induces a decrease in phagocytosis. We defined disease in lupus strains by the presence of anti-DNA antibodies, but we did not find a correlation between the anti-DNA titer and the clearance of apoptotic cells by macrophages. Further studies are needed to determine whether autoantibodies are involved, and if so which antibody specificity would then be responsible for inhibition of phagocytosis in diseased lupus mice.

Recently, several soluble factors have been shown to be required for the disposal of cellular and chromatin debris, and deficiencies in such factors can directly be linked to the etiology of SLE (13, 103). Targeted disruption of IgM (132, 134, 187) or C1q (124) leads to the development of antinuclear antibodies and glomerulonephritis. Disease development in these cases has been contributed to defective removal of apoptotic cells. Especially, complement has been shown to play

an important role, since C1q could bind to apoptotic cells and apoptotic cells could activate both the classical and the alternative pathway (125, 188). Also, impaired clearance of nucleosomes resulting from induced deficiency of DNaseI (131) or serum amyloid P (SAP) (129) causes SLE-like phenotypes characterised by antinucleosome antibodies and glomerulonephritis. Furthermore, thrombospondin (121), C-reactive protein (189), pentraxin (190), serum-derived protein S (191) and prothrombin (192) have also been implicated in the phagocytic clearance of apoptotic cells. Further studies are required to determine if any of the molecules mentioned above play a role in the decreased phagocytic efficacy of macrophages that we observed in diseased SLE mice. These studies could help to reveal which receptors and ligands are involved in the phagocytic clearance of apoptotic cells. This might suggest a number of targets (ligands, soluble bridging molecules, or receptors) that could be modulated to control exacerbations in SLE patients.

Chapter 5.

A prospective study of anti-nucleosome and anti-C1q auto-antibodies in patients with proliferative lupus nephritis treated with cyclophosphamide and azathioprine

Jürgen Dieker, Cecile Grootscholten, Fabian McGrath, Gerry Ligtenberg, Ron Derksen, Johan van der Vlag, Moh Daha & Jo Berden, on behalf of the Dutch Working Party on SLE

To be published

Abstract

The nucleosome is the major autoantigen in systemic lupus erythematosus (SLE). Anti-nucleosome autoantibodies, which comprise anti-DNA, anti-histone and nucleosome-specific autoantibodies, are specific for SLE and correlate with disease activity. Moreover, complexes of anti-DNA and anti-nucleosome autoantibodies with nucleosomes play an important role in the development of lupus nephritis, since they can bind to heparan sulfate in the glomerular basement membrane (GBM). Anti-C1q autoantibodies, which also correlate with disease activity, can bind to deposited C1q and via this mechanism can also bind to the GBM. We measured autoantibody levels in patients with proliferative lupus nephritis, who were treated with cyclophosphamide pulses, the standard treatment, or azathioprine in combination with methylprednisolone pulses. During the first year of treatment we found no difference between the two treatments for the levels of anti-nucleosome, anti-DNA, anti-histone and anti-C1q autoantibodies. We found a prevalence before starting treatment of 81%, 96%, 23% and 62% for anti-nucleosome, anti-DNA, anti-histone and anti-C1q autoantibodies, respectively. Anti-nucleosome correlated with anti-DNA and anti-histone, but not with anti-C1q levels. We found a correlation of anti-nucleosome, anti-DNA and anti-histone, but not anti-C1q, with disease activity measured by the SLEDAI. We found no significant differences for any of the antibodies between patients with high antibody reactivity or low antibody reactivity, for treatment efficacy, serological markers or renal function parameters. We conclude that almost all patients with proliferative lupus nephritis are positive for anti-nucleosome and/or anti-DNA autoantibodies, with no clinical differences between patients with high or low antibody titers. Treatment with cyclophosphamide or azathioprine induced a comparable decline in the levels of anti-nucleosome, anti-DNA and anti-C1q autoantibodies.

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the autoimmune response is autoantigen driven and T cell dependent. Historically, anti-double stranded (ds) DNA autoantibodies are the serological hallmark of the disease. However, dsDNA is not immunogenic by itself. Only bound to proteins, like histones or microbial proteins, dsDNA can induce an autoimmune response (9, 10). Therefore, the nucleosome has been proposed as the major autoantigen (13, 64). Nucleosomes are the basic structure of chromatin, which enable the dense packing of dsDNA in the nucleus. Each nucleosome consists of 146 bp of dsDNA wrapped twice around an octamer of pairs of the four core histones H2A, H2B, H3 and H4. Nucleosomes are the exclusive product of apoptosis, when chromatin is cleaved by DNases into individual nucleosomes. A defective removal of apoptotic cells leads to the release of (modified) nucleosomes, which can induce

an autoimmune response. Indeed, nucleosomes can be found in the circulation of SLE patients (49, 50). Moreover, histone- and nucleosome-specific T cells have been identified (54-56, 58). Anti-nucleosome autoantibodies can be divided into antibodies that recognize one of the components of the nucleosome, i.e. DNA or histones, and autoantibodies that preferentially recognize the intact nucleosome (nucleosome-specific antibodies). In lupus mice nucleosome-specific autoantibodies arise before anti-DNA autoantibodies (84). Anti-nucleosome are found in the vast majority of SLE patients (40-90%), especially in patients with lupus nephritis (193). High anti-nucleosome and also anti-DNA reactivity has been associated with disease activity and flares of lupus nephritis, one of the most serious manifestations of SLE (68, 70, 71, 75, 76, 79). The proportion of SLE patients which have anti-nucleosome antibodies, but no anti-DNA reactivity, which means the presence of nucleosome-specific antibodies, has been estimated around 12-30%, (71, 72, 79, 194). Especially, anti-nucleosome autoantibodies are specific for SLE, although some studies also report anti-nucleosome autoantibodies in patients with mixed connective tissue disease and scleroderma (68, 195). However, there are indications that the mucleosome preparation used in these studies, was contaminated with topoisomerase I, which might explain this reactivity (67). Anti-histone autoantibodies are less frequently found in SLE patients, but more often in patients with drug-induced lupus. However, their clinical relevance is still not clear (80). We regard the binding of anti-nucleosome/nucleosome complexes to the basement membrane in the glomerulus as a major event in the initiation of lupus nephritis (11). These complexes can bind to negatively charged heparan sulfate in the basement membrane via the positively charged histone tails, especially when anti-DNA or nucleosome-specific autoantibodies mask the negative charges on the nucleosomes (82).

Clearance of immune complexes involves the classical pathway of the complement system. Indeed, deficiency of members of the classical pathway, like C1q, C3 and C4, results in the development of anti-nuclear autoimmunity and glomerulonephritis in mice (124, 196). In patients deficient for C1q, SLE-like disorders are often found and about one third of the patients develop glomerulonephritis (197). Paradoxically, anti-C1q autoantibodies are also found frequently in SLE patients (30-45%) (198). Moreover, anti-C1q autoantibodies are especially found in patients with lupus nephritis and are predictive for renal flares (199, 200). Binding of anti-C1q/C1q complexes to the GBM can induce glomerulonephritis in mice (201), but is dependent on the presence of other IgG molecules (202). Moreover, anti-C1q autoantibodies might interfere with the function of C1q and thereby amplify the disease. Apart from its effect on the clearance of immune complexes, C1q is also involved in the removal of apoptotic cells, since mice with a targeted disruption of C1q have an impaired removal of

apoptic cells and develop anti-nucleosome reactivity and lupus-like glomerulonephritis (124).

Various studies, mainly conducted at the National Institute of Health (NIH) have indicated that treatment of lupus nephritis with a combination of corticosteroids and a cytotoxic immunosuppressive (either cyclophosphamide or azathioprine) provides better renal survival than treatment with corticosteroids alone (99). However, which cytotoxic immunosuppressive treatment should be given is still under debate. Intravenous cyclophosphamide pulses combined with oral steroids has been the standard treatment for proliferative lupus nephritis for many years, but side effects, in particular gonadal toxicity, are profound. Since most SLE patients are young females, an effective therapy associated with a reduced risk of infertility is warranted. Therefore, the Dutch Working Party on SLE initiated in 1995 a prospective randomized controlled trial to compare cyclophosphamide pulses with azathioprine and methylprednisolon pulses. Inclusion ended in 2001 and recently the first results after a minimum follow-up of 2 years were published (203).

The aim of this study is to assess the anti-nucleosome, anti-DNA, anti-histone and anti-C1q reactivity in these patients with proliferative lupus nephritis, treated with either cyclophosphamide combined with oral prednisone or azathioprine combined with methylprednisolone pulses and oral prednisone, during their first year of treatment. Autoantibody levels were analysed for their association with several serological markers, such as complement C3 and C4, disease activity and renal function parameters.

Materials & Methods

Patients

Eighty-seven patients with proliferative lupus nephritis were enrolled in the first Dutch Lupus Nephritis Study. All patients met the following criteria: presence of at least 4 American College of Rheumatology (ACR) criteria (61), age 18 to 60 years, creatinine clearance (Cockcroft-Gault formula (204)) >25 ml/min, and biopsy proven proliferative lupus nephritis according to the WHO-criteria (205). Patients were stratified according to center, serum creatinine (<150 μmol/l or >150 μmol/l), WHO-class III or IV, and previous treatment with cytotoxic immunosuppressive medication for lupus nephritis. Cyclophosphamide-treated patients (CY) were given six pulses of cyclophosphamide (750 mg/m^2) every 4 weeks, followed by seven pulses every 12 weeks, combined with oral prednisone, initially 1 mg/kg/day, tapered to a final dose of 10 mg daily after six months. Azathioprine-treated patients (AZA) started with azathioprine (2 mg/kg/day) at day one, combined with methylprednisolone intravenously (1000 mg) on three consecutive days. This cycle of three pulses was repeated after two and six weeks. In addition, oral prednisone (20 mg/kg/day) was given for five months and then tapered to 10 mg/day.

Plasma samples were collected before treatment and during the first year of treatment and stored at -80°C until use. Levels of complement components C3, C4 and anti-DNA titer (Farr assay and/or *Crithidia* test (206)) were measured. Overall disease activity was assessed by means of the SLE Disease Activity Index (SLEDAI) (207). A relapse was defined as doubling of the lowest obtained serum creatinine so far and/or development of either a nephrotic syndrome (proteinuria >3.5 g/day and serum albumin <30 g/l), while the lowest protein excretion so far had been >2.0 g/day repeatedly, or proteinuria >1.5 g/day without other causes, in a previously non-proteinuric patient. Complete remission was defined as proteinuria <1.0 g/day and a serum creatinine <130% of the lowest serum creatinine since start of treatment.

Anti-DNA, anti-histone and anti-nucleosome ELISA

All ELISA tests for autoantibody reactivity were repeated in a central laboratory. For the anti-nucleosome ELISA H1-stripped chromatin (kindly provided by Dr. R. Burlingame) was diluted 1:500 in PBS and 100 μl was coated overnight at 4°C in Maxisorb® 96-well plates (Nunc, Roskilde, Denmark). In the DNA ELISA, 100 μl of calf thymus dsDNA (Roche, Almere, The Netherlands) was coated overnight at 4°C in Maxisorb® 96-well plates at a concentration of 20 μg/ml in PBS.

In the histone ELISA 100 µl of calf thymus histones (Roche) were coated overnight at 4°C in a concentration of 2.5 µg/ml in 0.1 M glycine buffer at pH 9. The wells were washed with PBS/0.05% (v/v) Tween-20 (PBS-T) and blocked with 150 µl blocking reagent for 2 h at room temperature, i.e. 5% fetal calf serum (FCS) for the anti-DNA and anti-nucleosome ELISA and for the anti-histone ELISA 5% milk, which was treated with DNaseI, as previously described (97). After washing with PBS-T, 100 µl of the patient plasmas and positive and negative control plasmas were added at a starting dilution of 1:50 in blocking reagent and were incubated for 2 h at room temperature. After washing with PBS-T, 100 µl horseradish peroxidase-conjugated goat anti-human Ig(H+L) (Southern Biotechnology Associates, Birmingham, U.S.A.) diluted 1:10,000 in PBS-T was added and incubated for 1 h at room temperature. The plates were washed again with PBS-T and developed for 30 min using 100 µl 3,5,3',5'-tetramethylbenzidine (TMB) (SFRI Diagnostics, Saint Jean d'Ilac, France). The reaction was stopped by the addition of 100 µl 2 M H_2SO_4 and the optical density at 450 nm was measured in a microplate reader (Biorad). The titer (in arbitrary units (AU)) was defined as the dilution of the patient serum that yielded an absorbance of 0.5 at 450 nm. A standardized positive and negative patient plasma was used as standard in each experiment.

Anti-C1q ELISA

The anti-C1q ELISA was performed as described earlier (208). Briefly, Maxisorb® plates were coated with purified C1q at a concentration of 3 µg/well for 2 h at 37°C. After washing with PBS-T, a-specific binding was blocked with PBS/0.01% gelatin for 1 h at 37°C. After washing with PBS-T, samples were diluted in PBS-T with 1% fetal calf serum (FCS) and 1 M NaCl, added to the wells and incubated for 1 h at 37°C. Wells were washed with PBS-T and an DIG-labeled anti-human IgG diluted 1:10,000 in PBS-T/FCS was added. After washing with PBS-T, an HRP-conjugated anti-DIG antibody (Dako, Roche) diluted 1:8,000 in PBS-T/FCS was added. Plates were washed again with PBS-T and developed with 2,2'-Azino-bis-(3-ethylbenziazoline-6-sulfonic acid (ABTS)/H_2O_2 for 30 min. Optical density was measured at 415 nm in a microplate reader. Aserum positive for anti-C1q was used as a standard in each assay, plate reactivity was assessed as relative to the standard serum set at 100 aU/ml.

Statistics

Statistical analysis was performed using the Statistical Pack for Social Sciences version 12.0.1 (SPSS; Chicago, IL, USA). Comparisons of patient groups were performed by repeated measurements mixed model tests. The correlations

were determined by Spearman's rank correlation or chi-square test where appropiate. A p-value, after Bonferroni correction for multiple comparisons, of <0.01 was regarded statistically significant,

Results

Autoantibodies during first year of treatment

Autoantibodies were measured in ELISA in plasma's from patients with proliferative nephritis before treatment and during treatments after 4, 12, 26 and 52 weeks. Anti-nucleosome reactivity above the cut-off value (negative control+3xSD: 18.5 AU) was present in 81% of all the patients before treatment with no significant difference between the CY and AZA groups (80% vs 82%). Before starting treatment, anti-DNA and anti-histone reactivity above cut-off values (negative control+3xSD: 26.5 AU and 17.5 AU, respectively) were found in 96% and 23% of the patients, respectively, with also no significant difference between the CY and AZA groups. Anti-C1q reactivity (above 75 AU) was present in 62% of all patients before treatment. During treatment the median anti-nucleosome, anti-DNA and anti-histone reactivity showed no significant difference between both treatment groups

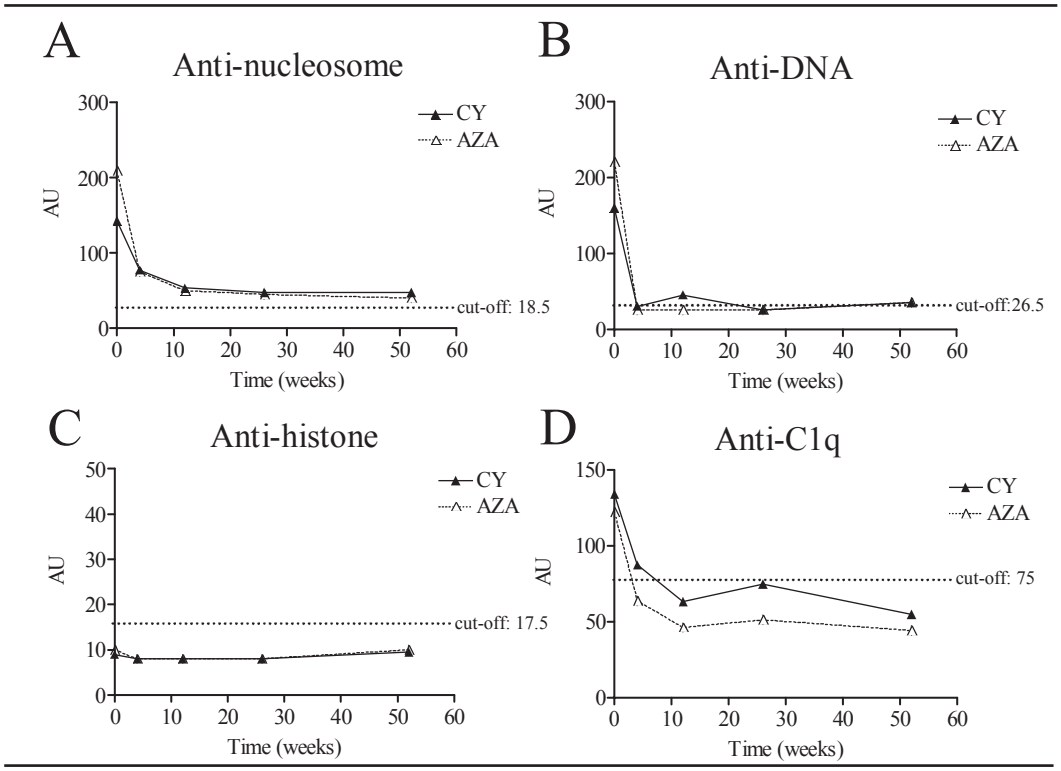

Figure 1. Autoantibody reactivity in CY and AZA-treated patients during the first year of treatment. Anti-nucleosome (**A**), anti-DNA (**B**), anti-histone (**C**) and anti-C1q (**D**) reactivity. Results are expressed as medians, AU=arbitrary units. The cut-off value for the different assays is indicated by the dashed lines.

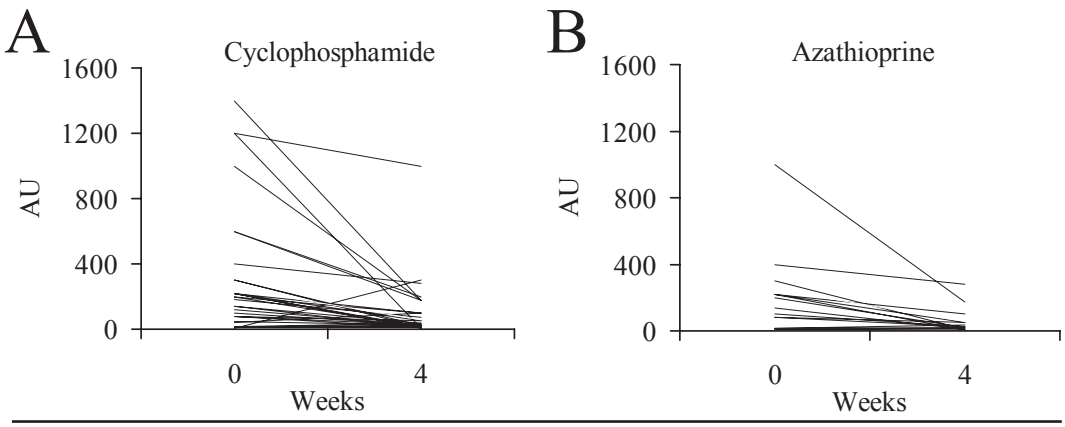

Figure 2. Decrease of anti-nucleosome antibody titers in CY (**A**) and AZA-treated (**B**) patients during the first 4 weeks of treatment. No statistically significant differences were found between the two treatment groups. AU=arbitrary units.

(Fig. 1). The reactivity against nucleosomes and dsDNA declined rapidly after treatment (Fig. 1, A and B). However, anti-histone reactivity was initial low and did not change during treatment (Fig. 1C). The anti-C1q reactivity decreased significantly during treatment, with no significant difference between the treatment protocols (Fig. 1D). Although the reactivity for anti-nucleosome, anti-DNA and anti-C1q declined during the first year of treatment, reactivity at 52 weeks stayed above the cut-off value in most patients. Since the two treatment regimens might have a different effect on the kinetics of the antibody titers, we compared the decrease of the titer of anti-nucleosome antibodies during the first 4 weeks of treatment (Fig. 2). We could not demonstrate a significant difference in the rate of decline between the CY and AZA treatment groups. We found comparable results for the decline of anti-DNA and anti-C1q titers (data not shown).

Correlation between autoantibodies

Since no significant differences were observed in titers and time course of the autoantibodies in CY and AZA treated patients, all patients were considered together for correlative calculations. Before treatment, anti-nucleosome reactivity showed a significant correlation with anti-DNA reactivity (r=0.629, Table I). We also found a significant correlation of anti-nucleosome and anti-DNA with anti-histone autoantibodies (r=0.583 and r=0.495, respectively). This underlines the fact that chromatin is the driving autoantigen in the formation of these autoantibodies. In contrast, anti-C1q reactivity showed no significant correlation with any of the anti-chromatin autoantibodies.

Table I. Correlation coefficients between autoantibody titers at the start of treatment

	Anti-DNA	Anti-histone	Anti-C1q
Anti-nucleosome	**0.629**	**0.583**	0.109
Anti-DNA		**0.495**	0.267
Anti-histone			0.115

Values in bold are significant ($p<0.01$)

Table II. Correlation coefficients of autoantibody titers with serological markers and disease activity before treatment

	Anti-nucleosome	Anti-DNA	Anti-histone	Anti-C1q
Farr	**0.491****	**0.531****	**0.361****	0.200
C3[1]	-0.241	**-0.418****	-0.231	-0.183
C4[2]	-0.057	-0.227	-0.088	0.024
SLEDAI[3]	**0.288***	**0.420****	**0.358***	0.165

*$p<0.01$ **$p<0.0001$

[1]C3: complement C3
[2]C4: complement C4
[3]SLEDAI: SLE disease activity index

Correlation of autoantibodies with other serological markers and disease activity

We analysed the correlation between the autoantibody titers before treatment measured in ELISA with the results of the Farr assay, which is often used as the standard assay to determine anti-DNA autoantibodies in these patients (Table II). Anti-nucleosome, anti-DNA and anti-histone reactivity showed a significant correlation ($p<0.0001$) with anti-DNA reactivity measured in the Farr assay. Anti-C1q reactivity showed no such correlation. Complement C4 levels showed no correlation with any of the autoantibodies, while C3 showed only a significant correlation with anti-DNA autoantibody levels (Table II). Finally, disease activity as assessed by the SLEDAI, correlated with anti-nucleosome, anti-DNA and anti-histone reactivity. We found no significant differences for the SLEDAI and anti-DNA measured by the Farr assay, when we compared patients with high (above the median) or low (below the median) anti-nucleosome, anti-DNA, anti-histone or anti-C1q antibody levels in ELISA (Tabel III).

We observed during the first year, an increase of anti-nucleosome and/or anti-nucleosome titers of more than 100%, in 8/52 patients. Only one of these 8 patients experienced a clinical renal relapse. Interestingly, in 6 of these 8 patients the doubling of the antibody titer occurred directly after induction of remission (data not shown). In the 2 other patients remission occurred after 56 weeks, while the increase on antibody titers was observed after 12 weeks. High anti-DNA and anti-

Table III. Characteristics of patients with high (above median) or low (below median) autoantibody levels

	Anti-nucleosome		Anti-DNA		Anti-histone		Anti-C1q	
	High	low	high	low	High	low	high	Low
	N=31	N=21	N=31	N=21	N=12	N=40	N=25	N=27
Treatment								
CY[1]	16	14	17	13	5	25	14	16
AZA[1]	15	7	13	9	7	15	11	11
Serological markers								
Anti-DNA (Farr) u/ml	768	128	737	141	1000	359	642	381
	(3-6940)	(0-716)	(13-6940)	(0-716)	(0-6940)	(3-3000)	(10-6940)	(0-3000)
C3 (mg/dl)	0.55	0.46	0.55	0.46	0.49	0.53	0.60	0.49
	(0.2-1.2)	(0.8-1.0)	(0.2-1.2)	(0.2-1.0)	(0.2-0.9)	(0.2-1.2)	(0.2-1.2)	(0.2-1.2)
C4 (mg/dl)	0.15	0.11	0.14	0.11	0.12	0.15	0.18	0.12
	(0-0.7)	(0-0.3)	(0-0.7)	(0-0.3)	(0-0.5)	(0-0.7)	(0.1-0.7)	(0-0.5)
Antibody flare[1,2]	6	2	7	1	4	4	4	4
Renal parameters								
GFR[3] (ml/min)	71	67	70	69	74	68	68	71
	(35-160)	(18-167)	(24-160)	(18-167)	(35-109)	(18-167)	(18-167)	(35-160)
Serum creatinin	123	144	127	138	116	136	149	116
(µmol/L)	(58-227)	(64-472)	(58-227)	(64-472)	(67-206)	(58-472)	(58-472)	(64-227)
Proteinuria	3.8	4.4	3.7	4.5	4.7	3.9	4.4	3.7
(g/24h)	(0.1-8.4)	(1.2-9.2)	(0.1-8.5)	(1.2-9.2)	(0.1-8.5)	(0.5-9.2)	(1.7-8.5)	(0.1-9.2)
Disease activity								
Failure/relapse/death[1]	7	4	7	4	4	7	7	4
Complete remission[1]	20	15	20	15	8	27	16	19
Time to complete	29.2	26.9	29.2	26.7	24.7	29.3	26.1	30.2
remission (weeks)	(0-64)	(4-62)	(2-64)	(0-62)	(2-60)	(0-64)	(2-64)	(0-56)
Primary nephritis[1]	6	6	9	3	1	11	6	6
SLEDAI	20	17	21	16	22	18	20	18
	(8-38)	(6-26)	(8-38)	(6-26)	(8-38)	(6-33)	(8-38)	(6-34)

[1] number of patients
[2] increase (>100%) in anti-nucleosome and/or anti-DNA antibody titers during first year
[3] calculated GFR with the Cockroft-Gault formula

CY: cyclophosphamide
AZA: azathioprine
GFR: glomerular filtration rate

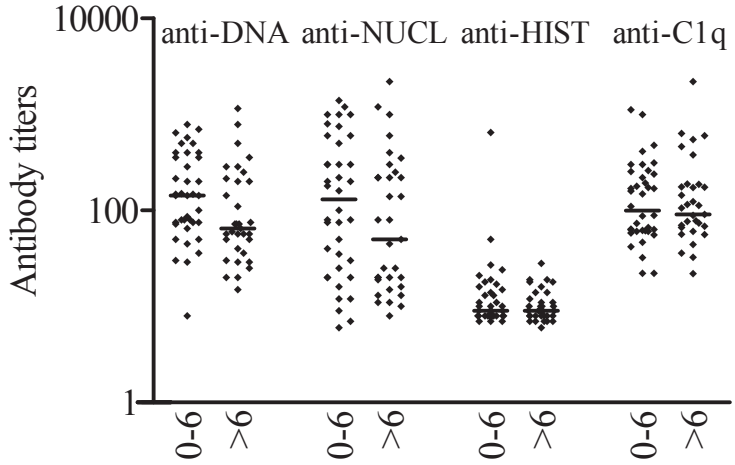

Figure 3. Antibody titers in patients with remission within 6 months or remission after 6 months. No statistical significant differences were found.

nucleosome titers at the start of treatment, were predictive for the doubling of antibody titers later on during ther first year (7 out of 8 and 6 out of 8 respectively; Table III).

Association with renal function parameters

We compared renal function parameters in patients with high levels (above the median) of anti-nucleosome, anti-DNA, anti-histone or anti-C1q autoantibodies with those in patients with low (below the median) levels (Table III). We found no significant difference between patients with high or low autoantibody levels for the calculated GFR, serum creatinine or proteinuria. We compared the course of the disease in patients with high versus low antibody titers and found no significant difference for the occurrence of failure, relapse, death, or complete remission. Moreover, we could not demonstrate a significant difference in the time to complete remission. In addition, we compared antibody titers in patients with remission within 6 months or remission after 6 months (Fig. 3). No significant difference could be demonstrated. We also looked at patients for whom this was the primary nephritis compared to patients who were already experienced nephritis before. We found no significant difference in antibody levels (data not shown), but patients with primary nephritis tended to have higher anti-DNA and lower anti-histone autoantibody reactivity, but this difference did not reach statistical significance (Table III). Although the SLEDAI was for all autoantibody specificities numerically higher in the high titer group, this difference was not statistically significant.

Discussion

In experimental animal models complexes of anti-nucleosome or anti-DNA with nucleosomes, possibly in combination with anti-C1q/C1q complexes, play a decisive role in the development of lupus nephritis (82). Moreover, these autoantibodies are correlated with the occurence of lupus nephritis (70). Although many studies have described the prevalence, sensitivity and specificity of these autoantibodies in SLE patients (68, 70, 193), not much is known about the characteristics of these autoantibodies and their relation to clinical parameters in patients with a proliferative lupus nephritis. We showed a high prevalence of anti-DNA and anti-nucleosome autoantibodies in these patients (96% and 81%, respectively). The prevalence of anti-DNA and anti-nucleosome was higher than is found on average in unselected SLE patients. This can be expected since the presence of these autoantibodies correlates with the occurrence of lupus nephritis (72, 76). Remarkably, in our cohort the prevalence of anti-DNA autoantibodies is higher than the presence of anti-nucleosome autoantibodies. This has been found before (71, 209). SLE patients which are only positive for anti-DNA autoantibodies have been described (72, 194). Based on our results we can not prove the anticipated superiority of anti-nucleosome testing in these patients.

We found a correlation between anti-DNA and anti-nucleosome, which is comparable to values found in the total group of SLE patients (70, 71, 75, 76, 210). Prevalence of anti-histone autoantibodies was low (23%). Moreover, anti-histone reactivity was low in the majority of the patients. Indeed, anti-histone autoantibodies are more often found in drug-induced lupus (80), and do not correlate with renal disease (193). Studies in mice showed that complexes of monoclonal anti-histone autoantibodies and nucleosomes bound less efficient to the glomerular basement membrane (GBM), possibly by masking of the positive histone charges involved in the GBM-binding by the antibody (82).

Anti-C1q autoantibodies are also associated with lupus nephritis (198). We found that 66% of the patients showed positieve anti-C1q reactivity, which is comparable to reported prevalences in patients with lupus nephritis (198, 211). No correlations were reported between anti-chromatin antibodies and anti-C1q autoantibodies before treatment, which can be explained by a different inducer (nucleosomes and C1q) of the immune responses.

We compared the standard treatment of proliferative lupus nephritis with cyclophosphamide to an alternative treatment with azathioprine/ methylprednisolone. No differences were found in the induction of remission although relapses occurred more frequently in the AZA treated patients (203). In addition, we found in this study no differences on the course of autoantibody titers

during the first year of treatment between the two treatment groups. Autoantibody titers declined very fast after start of treatment, although anti-DNA tended to declined somewhat faster than anti-nucleosome autoantibodies. To evaluate this in more detail we compared the kinetics of this decline, from week 0 to week 4 for both treatments. However, this revealed no difference for these autoantibody specificities.

Anti-nucleosome and anti-DNA autoantibodies have been associated with disease activity and renal function in SLE patients (70, 193). However, these correlations were investigated in patients with and without lupus nephritis. Anti-nucleosome, anti-DNA and anti-histone autoantibodies showed a positive correlation with the SLEDAI, comparable to the literature (70, 71, 79). Remarkable was the absence of such a correlation with anti-C1q reactivity. Although anti-nucleosome and anti-C1q autoantibodies have been shown by others to correlate with the presence of lupus nephritis (68, 71, 193, 211), we could not observe in our patient cohort a direct correlation between anti-nucleosome and anti-C1q autoantibody titers and markers for renal function (i.e. GFR, serum creatinine, proteinuria). In addition, we found no difference in renal function parameters between patients with high autoantibody titers compared to patients with low autoantibody titers. The absence of these correlations might be due to the fact that all our patients had a biopsy proven lupus nephritis with more or less comparable disease manifestations. Significant increases in autoantibody titers (i.e. anti-nucleosome and/or anti-DNA) during the first year of treatment were more frequent observed in patients with high anti-nucleosome or anti-DNA at the start of treatment. We also looked whether patients who were only positive for one autoantibody specificity (either anti-nucleosome of anti-DNA) were different for clinical parameters from patients who were positive for both specificities. However, this showed comparable results (data not shown).

In conclusion, we have shown here that treatment of proliferative lupus nephritis with azathioprine does not result in significant differences in anti-chromatine or anti-C1q autoantibody kinetics compared to patients treated with cyclophosphamide. In addition, the vast majority of lupus nephritis patients are positive for anti-nucleosome, anti-DNA and/or anti-C1q autoantibodies. Their titers correlate with disease activity, but not with renal function parametes or disease course. These findings imply that measurement of these autoantibody specificities are important tools for diagnosis of lupus nephritis but not for monitoring treatment.

Acknowledgements

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Chapter 6.

Mimotopes for lupus-derived anti-DNA and nucleosome-specific autoantibodies selected from random peptide phage display libraries: facts & follies

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Abstract

Autoantibodies against chromatin are the most characteristic serological feature in SLE patients. Anti-DNA and nucleosome-specific antibodies are associated with glomerulonephritis, the most serious manifestation of SLE. Identification of peptides mimicking conformational epitopes (so-called mimotopes) on the nucleosome recognized by these antibodies is of considerable interest. Using an approach similar to that used previously to characterize mimotopes for anti-DNA autoantibodies, we have selected and identified a mimotope for a nucleosome-specific autoantibody (#32) by screening a random peptide phage display library. However, the reactivity of monoclonal antibody (mAb) #32 with the selected mimotope (MIMO#0) in ELISA was dependent on the blocking reagents used. Using nonfat dry milk (5%), mAb #32 clearly bound to MIMO#0, but using fetal bovine calf serum (FCS) (5%), there was no binding. Furthermore, again dependent on the blocking reagent used in ELISA, the selected mimotope MIMO#0 was not only recognized by the selecting antibody mAb #32, but also by a large number of other monoclonal anti-DNA, anti-histone and nucleosome-specific autoantibodies (NSA). We could demonstrate that the selected mimotope was able to bind directly to nucleosomal material (DNA/histone complexes) and labeled DNA. This finding was extended to other previously identified mimotopes for anti-DNA autoantibodies. We conclude that nucleosomal material (DNA/histone complexes), derived from reagents used during the mimotope selection procedure, resulted in the selection of DNA-binding peptides from the phage display library, rather than mimotopes. In addition, we could demonstrate that blocking reagents greatly influence the reactivity of anti-DNA, anti-histone and nucleosome-specific autoantibodies in ELISA. Development of blocking reagents devoid of nucleosomal material (DNA/histone complexes) is urgently needed for assay systems in which anti-nuclear autoantibodies are tested.

Systemic lupus erythematosus is an autoimmune disease, in which the autoimmune response is T cell dependent and autoantigen-driven (99). Autoantibodies detected in lupus patients are directed against a variety of autoantigens, particularly against chromatin. Basically, chromatin consists of a chain of nucleosomes, which are octamers of the histones H2A, H2B, H3 and H4, around which 146 base pairs of double stranded DNA (dsDNA) are wrapped, while histone H1 is bound to the linker DNA between the nucleosomes. Anti-chromatin autoantibodies can be divided into those antibodies directed against one of the subcomponents of the nucleosome, either DNA or histones, and those antibodies which only recognize the intact nucleosome (so-called nucleosome-specific antibodies). Historically, the serological hallmark of SLE was considered to be the presence of anti-DNA autoantibodies (100), but now the presence of nucleosome-

specific antibodies appears to be a better marker (12, 13). Nucleosome-specific antibodies have been found in 88% of SLE patients, and correlate with disease activity (64). Initially, it was claimed that they were lupus specific (70), but later it has been reported that they can also be found in other autoimmune diseases, such as mixed connective tissue disease and systemic sclerosis (68). Identification of the conformational epitopes recognized by nucleosome-specific autoantibodies could provide more insight into the pathogenesis of SLE. This approach may also reveal important tools for both diagnostic and therapeutic applications. Several epitopes for anti-histone autoantibodies and histone-specific T cells have been identified (58, 212-214). However, the identification of epitopes for anti-DNA or nucleosome-specific antibodies requires another approach, since these epitopes also encompass non-protein structures (i.e. DNA). Screening of random peptide phage display libraries, in which random peptides are expressed on one of the coat proteins of the phage, with monoclonal antibodies (mAb) against non-protein structures such as DNA has revealed peptides, so called mimotopes, which mimic the non-protein structures recognized by the antibody, such as anti-DNA antibodies (87-89, 215).

In this study, we identified a nucleosome-specific mimotope by screening a 10-mer random peptide phage display library with a nucleosome-specific monoclonal autoantibody. Surprisingly, this mimotope appeared not to be specific and, depending on the applied blocking reagent in the ELISA, it was not only recognized by the selecting antibody, but also by many other anti-chromatin autoantibodies. More importantly, the reactivity in ELISA of the antibody used for selection with the mimotope peptide was dependent of the blocking reagent used. We found in ELISA the same blocking-dependent variation in reactivity of anti-chromatin antibodies with a previously identified DNA mimotope peptide and a histone peptide. Further analysis revealed that this blocking-dependent variation in poly-reactivity with the three peptides was caused by nucleosomal material present in the blocking reagents used in the ELISA procedure. Due to the presence of nucleosomal material derived from reagents used during selection of phages, DNA binding peptides rather than nucleosome-specific mimotopes were selected.

Materials & Methods

Antibodies

The anti-chromatin monoclonal autoantibodies used in this study are listed in Table I. All antibodies were purified on a protein A Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) under high salt conditions after DNaseI treatment of the culture supernatant (94). Free histones and histone-complexed antibodies were removed on a DNA-cellulose column (Amersham Pharmacia Biotech) as described previously (94). The purity of the antibodies (i.e. absence of histones and DNA) was checked by SDS-PAGE analysis followed by silver staining (Bio-Rad Laboratories, Veenendaal, The Netherlands). The presence of DNA was identified by extraction with phenol chloroform, separation and analysis on a 1% agarose gel using ethidium bromide staining. Other monoclonal control antibodies used in this study were WT-32, an IgG2a anti-CD3 antibody (216), and anti-MYC (Invitrogen, Breda, The Netherlands). Human sera used in this study were collected from SLE patients at the National University Hospital, Singapore, and tested for the presence of antibodies to dsDNA as described earlier (89).

Table I. Characteristics of the monoclonal anti-chromatin autoantibodies used in this study.

mAb	Specificity[a)]	Source	Isotype	Reference
NZA2	Nucleosome	NZB	IgG2aκ	(219)
#32	Nucleosome	(NZWxNZB)F1	IgG2aκ	(81)
LG10-1	Nucleosome	MRL/lpr	IgG2bκ	(219)
PL2-3	H2A/H2B-DNA	MRL +/+	IgG2aκ	(220)
#34	H3	(NZWxNZB)F1	IgG2aκ	(81)
KM-2	H2A/H4	MRL/lpr	IgG2aκ	(95)
#42	dsDNA	(NZWxNZB)F1	IgG2aκ	(81)
#36	dsDNA	(NZWxNZB)F1	IgG2aκ	(81)

[a)]As determined in ELISA as described previously (221)

Blocking reagents

Blocking reagents used in this study were: 5% nonfat dry milk (Bio-Rad Laboratories), 5% fetal bovine calf serum (FCS) (Invitrogen), 1% bovine serum albumin (BSA) purity 96%, 98% and 99%, 1% casein, 1% fish gelatin (all from Sigma, Zwijndrecht, The Netherlands) and 1% Aurora Blocking Reagent (Perbio Science, Etten-Leur, The Netherlands).

Biopanning of peptide library by nucleosome-specific antibody #32

The L100 library, displaying a 10-mer random peptide on phage coat-protein III, kindly provided by Dr. M. Scharff (Einstein College, New York), was screened with nucleosome-specific mAb #32. Library screening was performed as described previously (217). Briefly, in the first panning round 1 μM of mAb #32 (belonging to the IgG2a subclass) and 2 μM of biotin-labeled goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, USA) were incubated and subsequently added to 50 μl streptavidin-coated magnetic beads (Qiagen, Hilden, Germany). The antibody-coated streptavidin beads were incubated for 4 h with an aliquot of the phage library containing 10^{12} transducing units (TU) in 100 μl of biopanning buffer (150 mM NaCl/10 mM Tris–HCl pH 7.4/0.1% (v/v) Tween-20/0.1% (w/v) BSA). Beads were collected by a magnet (Polysciences, Warrington, USA), and unbound phages were removed by washing with biopanning buffer. Phages were eluted with 100 μl of 0.1 M glycine-HCl (pH 2.2) for 10 min and directly neutralized with 16 μl 2 M Tris–HCl pH 8.0. Eluted phages were amplified overnight in Escherichia coli K91kan in LB medium with 20 μg/ml tetracycline and 100 μg/ml kanamycin. Amplification of phages, PEG purification and phage titration were performed as described previously ((218). For the second and third round of panning, either 1 μM and 10 nM or 10 nM and 100 pM of mAb #32, respectively, were incubated for 4 h with phages from the previous round containing 10^{7}–10^{10} TU. Next, the phage/antibody complexes were captured shortly for 10 min by 50 μl (1 μM antibody) or 10 μl (other concentrations) streptavidin-coated magnetic beads saturated with biotin-labeled goat-anti mouse IgG2a antibody. Individual clones were picked after three rounds of selection and grown overnight in LB medium containing 20 μg/ml tetracycline and 100 μg/ml kanamycin. Phage ssDNA was isolated with the Qiaprep Spin M13 kit (Qiagen), according to the protocol supplied by the manufacturer, and sequenced according to the Sanger method by the BigDye Terminator (Applied Biosystems), using an 22-base fUSE5 anti-sense primer (5'-gtaacgatctaaagttttgtcg-3').

Peptides

Peptides were synthesized by Genemed Synthesis (San Francisco, USA) on an eight-branched multi-antigen peptide (MAP) backbone. The purity was >70%, as checked by HPLC. The MAP-peptides were dissolved in DMSO at a concentration of 100 mg/ml. MAP-peptides used for this study were: MAPMIMO#0 (MAP-GSGGNRKDWIERTLGAPS), a mimotope for nucleosome-specific monoclonal autoantibody #32; MAP-DNAmimic (MAP-RLTSSLRYNP), a mimotope for polyclonal anti-DNA autoantibodies, and variants of this peptide used for the alanine scanning (MAP-ALTSSLRYNP, MAPRATSSLRYNP, MAP-RLASSLRYNP, etc.); MAP-H3(21-37) (MAP-ATKAARKSAPATGGVKK), recognized by anti-histone antibody #34, and MAP-MYC (MAP-GSGGEQKLISEEDLGAPS), containing a MYC-tag sequence. MAP-MIMO#0 and MAP-MYC contained the recognized motif (underlined) and four flanking residues derived from the phage coat-protein.

Biotin labeling of DNA

Calf thymus DNA was labeled with biotin using the nick translation mix (Roche). Briefly, 10 µg of DNA were incubated with 4 µl nick translation mix in a total volume of 20 µl for 1 h at room temperature. The reaction was stopped by adding EDTA to a final concentration of 15 mM.

Peptide ELISA

Maxisorb® 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 µl of peptide at a concentration of 25 µg/ml in 50 mM carbonate buffer pH 9.6 overnight at 4°C. The wells were washed with PBS/0.05% (v/v) Tween-20 (PBS-T) and blocked with 150 µl blocking reagent for 2 h at room temperature. After washing with PBS-T, 100 µl of monoclonal antibody were added at a concentration of 100 ng/ml in PBS-T and incubated for 2 h at room temperature. After washing with PBS-T, 100 µl horseradish peroxidaseconjugated goat anti-mouse Ig(H+L) (Southern Biotechnology Associates) diluted 1:7500 in TBS-T were added and incubated for 1 h at room temperature. The plates were washed again with PBS-T and developed for 15 min using 100 µl 3,5,3',5'-tetramethylbenzidine (TMB) (SFRI Diagnostics, Saint Jean d'Ilac, France). The reaction was stopped by the addition of 100 µl 2 M H_2SO_4 and the absorbance at 450 nm was measured in a microplate reader (Biorad). The results for the various types of anti-chromatin autoantibodies (i.e. anti-DNA, anti-histone and nucleosome-specific) are depicted for each class of autoantibodies as mean (±S.D.) of the absorbance. For the binding of biotin-labeled DNA, 0.5 µg of biotin-labeled DNA was added in 100 µl PBS-T after the blocking

step and incubated for 30 min at room temperature. After washing with PBS-T, 100 µl of horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech) diluted 1:500 in PBS-T were added for 1 h at room temperature. Development and measurement of the absorbance was performed as described above.

DNaseI treatment of blocking reagents

Blocking reagents were incubated with 20 µg/ml of grade II DNaseI (Roche) and 1 mM $MgCl_2$ for 30 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 15 mM.

SDS-PAGE and immunoblotting

A range of dilutions of blocking reagent dissolved in PBS was added to 2 volumes of 3x Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 5% β-mercaptoethanol), and was heated for 5 min at 95°C and resolved by 18% SDS-PAGE gel electrophoresis using the Protean III System (Bio-Rad Laboratories). The resolved proteins were transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories) in blotting buffer (125 mM glycine, 25 mM Tris, 0.02% SDS and 20% methanol). The blots were washed in PBS and blocked in 1%(w/v) blocking reagent (Roche) for 1 h. After washing with PBS (3x) and phosphate buffered solution containing Tween/fish gelatin (Sigma)/sodium (PBTGNa) (0.1% (w/v) gelatin, 0.5% (w/v) BSA, 0.05% (v/v) Tween-20 and 300 mM NaCl in PBS) (1x), blots were incubated for 2 h with monoclonal anti-histone antibody KM-2, diluted 1:100 in PBTGNa. After washing with PBS (3x) and PBTGNa (1x), blots were incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse Ig(H+L) (Southern Biotechnology Associates), diluted 1:7500 in PBTGNa. After washing three times with PBS, blots were developed using LumiLight® (Roche) and exposed to Hyperfilm ECL X-ray films (Amersham Pharmacia Biotech).

Results

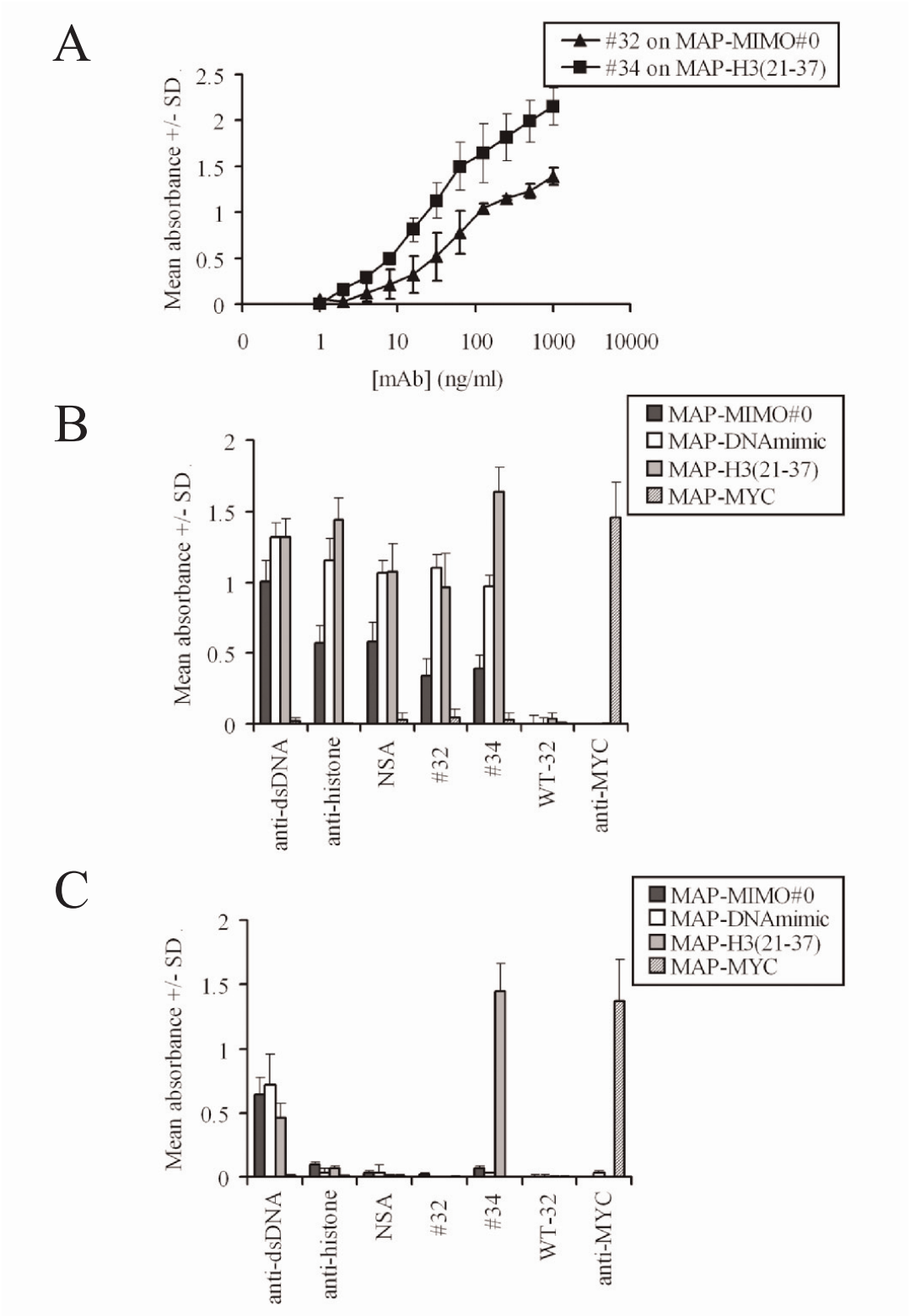
Selection of a mimotope for a nucleosome-specific antibody

The nucleosome-specific mAb #32 was used to screen a 10-mer random peptide phage display library. Three rounds of selection were performed using a decreasing concentration of antibody during the panning rounds (1 μM, 10 nM and 100 pM, respectively). Positive clones were selected after the third round of panning with 100 pM antibody and the common amino acid motif NRKDWIERTL was deduced from the DNA sequence of 10 separate clones. To confirm *in vitro* binding of mAb #32 in ELISA, a peptide containing the motif NRKDWIERTL (MAP-MIMO#0) was synthesized on an eight-branched multiantigen peptide (MAP) backbone with four flanking amino acids of the pIII phage coat-protein, which resembles the amino acid context during selection. Antibody #32 showed reactivity with this MAP-synthesized MIMO#0 in ELISA (Fig. 1A).

Poly-reactivity of anti-chromatin autoantibodies

Surprisingly, other nucleosome-specific autoantibodies (NSA), but also anti-DNA and anti-histone autoantibodies showed reactivity with the MAP-MIMO#0 peptide (Fig. 1B). The poly-reactivity of a wide range of anti-chromatin autoantibodies was not unique to the mimotope we identified for the nucleosome-specific autoantibody #32, since a similar broad reactivity was found with a previously described DNA mimicking MAP-peptide (RLTSSLRYNP, hereafter designated as the MAP-DNAmimic). This DNA mimicking peptide was selected by

Figure 1. (next page) **A.** Reactivity of nucleosome-specific autoantibody #32 with MAP-MIMO#0, selected with this antibody, and anti-histone autoantibody #34 with MAP-H3(21-37) in ELISA. (**B**, **C**) Poly-reactivity of anti-chromatin autoantibodies. Nucleosome-specific (NSA: i.e. NZA2, LG10-1 and PL2-3), anti-histone (i.e. KM-2) and anti-DNA (i.e. #36 and #42) autoantibodies, nucleosome-specific autoantibody #32, anti-histone autoantibody #34 or control antibodies (WT-32 and anti-MYC) were tested at a concentration of 100 ng/ml with MAP-MIMO# 0, MAP-DNAmimic, MAP-H3(21-37) and control peptide MAP-MYC, using nonfat dry milk (**B**)or FCS as blocking reagent (**C**) All anti-chromatin antibodies showed reactivity with MAP-MIMO#0, MAP-DNAmimic and MAP-H3(21-37) using milk as blocking reagent. When FCS was used, only reactivity of the anti-DNA autoantibodies with these three peptides, and reactivity of anti-histone autoantibody #34 with MAP-H3(21-37) was observed. No reactivity of the anti-chromatin antibodies was found with the control peptide MAP-MYC. Coating of the MAP-peptide was appropriate as seen with the anti-MYC antibody. The control monoclonal antibody WT-32 did not bind to any of the peptides.


A
#32 on MAP-MIMO#0
#34 on MAP-H3(21-37)
Mean absorbance +/- SD
2.5
2
1.5
1
0.5
0
0
1
10
100
1000
10000
[mAb] (ng/ml)
B
MAP-MIMO#0
MAP-DNAmimic
MAP-H3(21-37)
MAP-MYC
Mean absorbance +/- SD
2
1.5
1
0.5
0
anti-dsDNA
anti-histone
NSA
#32
#34
WT-32
anti-MYC
C
MAP-MIMO#0
MAP-DNAmimic
MAP-H3(21-37)
MAP-MYC
Mean absorbance +/- SD
2
1.5
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0.5
0
anti-dsDNA
anti-histone
NSA
#32
#34
WT-32
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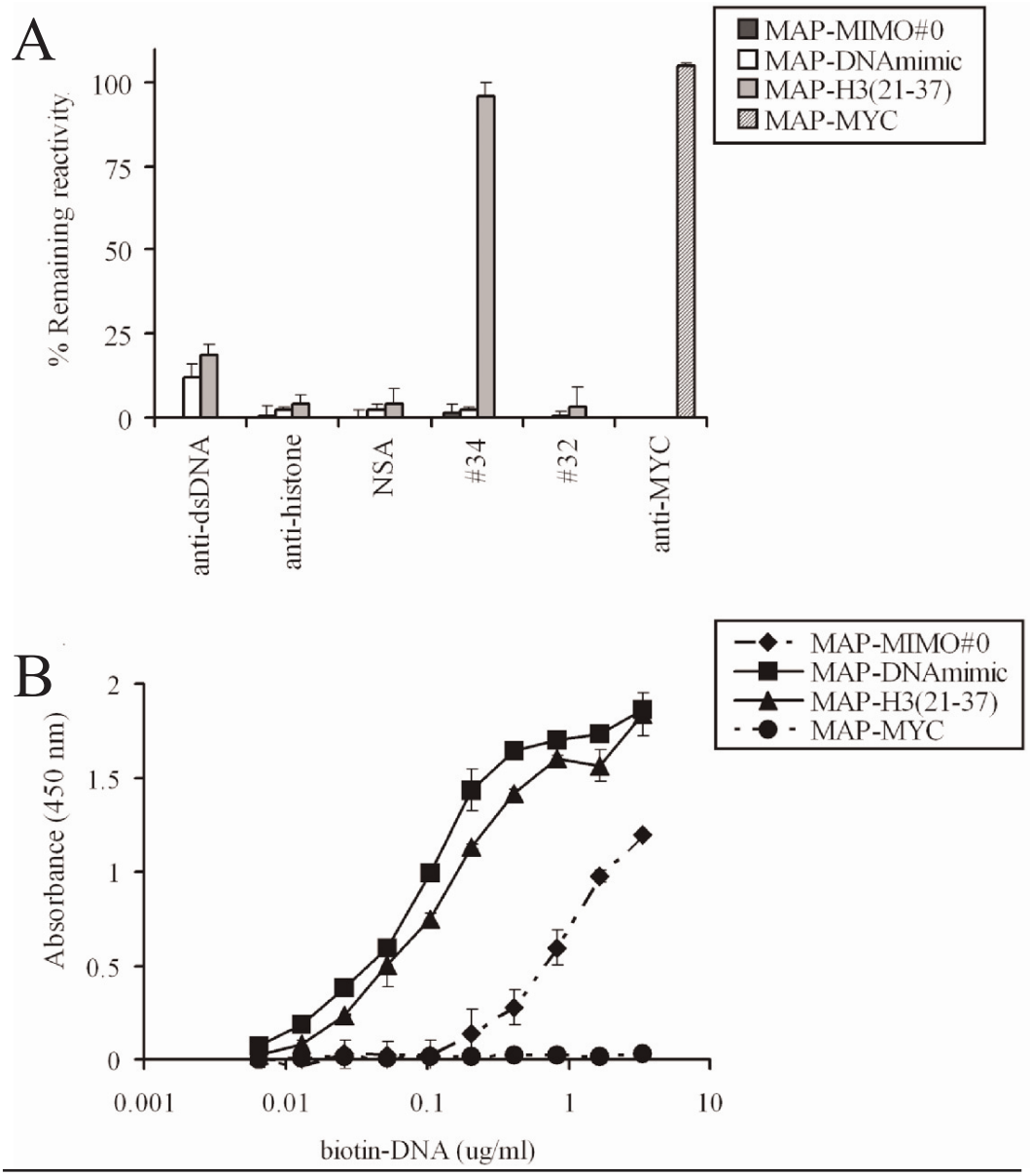

Figure 2. Effect of DNaseI treatment of the blocking reagent on the binding of anti-chromatin antibodies. **A.** DNaseI treatment of milk resulted in a dramatic decrease of the reactivity of all anti-chromatin antibodies with the MAP-DNAmimic, MAP-MIMO#0 and MAP-H3(21-37). Interactions of anti-histone autoantibody #34 with MAP-H3(21-37) and anti-MYC with MAP-MYC were not affected by the DNaseI treatment. Results are expressed as percentage residual reactivity calculated by dividing the reactivity after DNaseI treatment by the reactivity without DNaseI treatment, x100. **B.** Addition of biotin-labeled DNA to coated MAP-peptides after blocking with DNaseI-treated milk shows binding of dsDNA to MAP-MIMO#0, MAP-DNAmimic and MAP-H3(21-37). No DNA-binding was observed with the control peptide MAP-MYC. Results are expressed as absorbance values at 450 nm.

screening a 15-mer pVIII phage library with polyclonal anti-DNA antibodies isolated from SLE patient sera via a DNA-Sepharose column (89) (Fig. 1B). In addition, a histone peptide was also recognized by the set of anti-DNA, anti-histone and nucleosome-specific autoantibodies tested (Fig. 1B). This histone peptide, comprising the histone H3 amino acids 21–37 synthesized on a MAP backbone (MAP-H3(21-37)), contains the epitope of antibody #34 as shown before (221). The monoclonal anti-histone H3 autoantibody #34 showed a high reactivity with this peptide (Fig. 1A). The control antibodies WT-32 and anti-MYC showed no reactivity with either MAP-MIMO#0, MAP-DNAmimic or MAP-H3(21-37) (Fig. 1B). To exclude reactivity with the MAP backbone, all antibodies were tested with a control MAP-peptide, containing an MYC-tag sequence (Fig. 1B). None of the anti-chromatin autoantibodies bound to this control peptide. This was not due to insufficient coating of the MAP-MYC peptide, since an anti-MYC antibody showed good reactivity (Fig. 1B). Furthermore, coating of MAP backbone alone did not reveal binding of any of the antibodies tested (data not shown).

Influence of the blocking reagent

Since we could not directly explain the polyreactivity of our anti-chromatin autoantibodies with MAP-MIMO#0, MAP-DNAmimic and MAP-H3(21-37), we applied a different ELISA protocol using FCS instead of nonfat dried milk as blocking reagent. Surprisingly, completely different results were obtained when FCS was used as blocking reagent (Fig. 1C). Under these conditions, the poly-reactivity with the MAP-MIMO#0, MAP-DNAmimic and MAPH3(21-37) was restricted only to the anti-DNA autoantibodies, while the reactivity of the nucleosome-specific and anti-histone autoantibodies disappeared. Compared to the ELISA using milk as blocking reagent (Fig. 1B), the reactivity of the anti-MYC antibody with the MAP-MYC and the reactivity of anti-histone H3 autoantibody #34 with MAP-H3(21-37) were not affected by the presence of FCS (Fig. 1C). At first glance, this would suggest that MAP-MIMO#0 and the MAP-DNAmimic were mimicking DNA; but this does not explain why anti-DNA antibodies were found bind to the histone peptide MAP-H3(21-37). We also analyzed other blocking reagents, including BSA, casein, fish gelatin and Aurora Blocking reagent, and we found a comparable poly-reactivity with MAP-MIMO#0, MAP-DNAmimic and MAP-H3(21-37), as when using FCS as blocking reagent (data not shown). Most importantly, the reactivity of the selecting mAb #32 with its mimotope, MAP-MIMO#0, was dependent on the blocking reagent, i.e. positive binding using milk (Fig. 1B) and no binding using FCS (Fig. 1C).

Effects of DNaseI treatment

Since the antibodies that showed poly-reactivity were all anti-chromatin autoantibodies, the influence of the blocking reagent on the reactivity of these autoantibodies suggests that components of the nucleosome, i.e. DNA and/or histones, were present in the blocking reagent. We therefore performed a DNaseI treatment of nonfat dry milk, to determine what effect this had on the binding of anti-chromatin autoantibodies to the MAP-peptides (Fig. 2A). The poly-reactivity of anti-DNA, and also of nucleosome-specific and anti-histone autoantibodies, was completely lost after DNaseI treatment. Note that the reactivity of the antibody used for selection, i.e. #32, with MAP-MIMO#0, was almost completely lost upon DNaseI treatment of milk. The binding of autoantibody #34 with MAP-H3(21-37), and anti-MYC with MAP-MYC was not affected by DNaseI treatment of the blocking reagent.

Next, we added DNA, histones or nucleosomes to the DNaseI-treated blocking reagent to see if the poly-reactivity could be reconstituted. Indeed, the poly-reactivity of anti-DNA antibodies reappeared by the addition of 12 ng/ml calf thymus dsDNA after the blocking step. Furthermore, the addition of nucleosomes (concentration of 10 ng/ml dsDNA) after the blocking step could also restore the binding of anti- DNA, anti-histone and nucleosome-specific autoantibodies to the MAP-bound epitopes/mimotopes (data not shown).

Direct binding of DNA

The effect of DNaseI treatment on the binding of anti-DNA, and also anti-histone and nucleosome-specific autoantibodies to the MAP-peptides, suggested that DNA, present in nonfat dry milk, caused the binding of anti-chromatin autoantibodies. To show direct binding of dsDNA to all these peptides, i.e. MAP-MIMO#0, MAP-DNAmimic and MAP-H3(21- 37), we added biotin-labeled calf thymus dsDNA to the incubation medium (Fig. 2B). Biotin-labeled dsDNA was able to bind dose-dependently to all three MAP-peptides, but not to the MAP-MYC control peptide (Fig. 2B). Furthermore, the addition of biotin labeling mix without DNA resulted in no binding to any of the MAP-peptides (data not shown).

Presence of nucleosomal material in blocking reagents

The dependency of the poly-reactivity of anti-chromatin autoantibodies with MAP-peptides on the blocking reagent, and the effect of DNaseI treatment of the

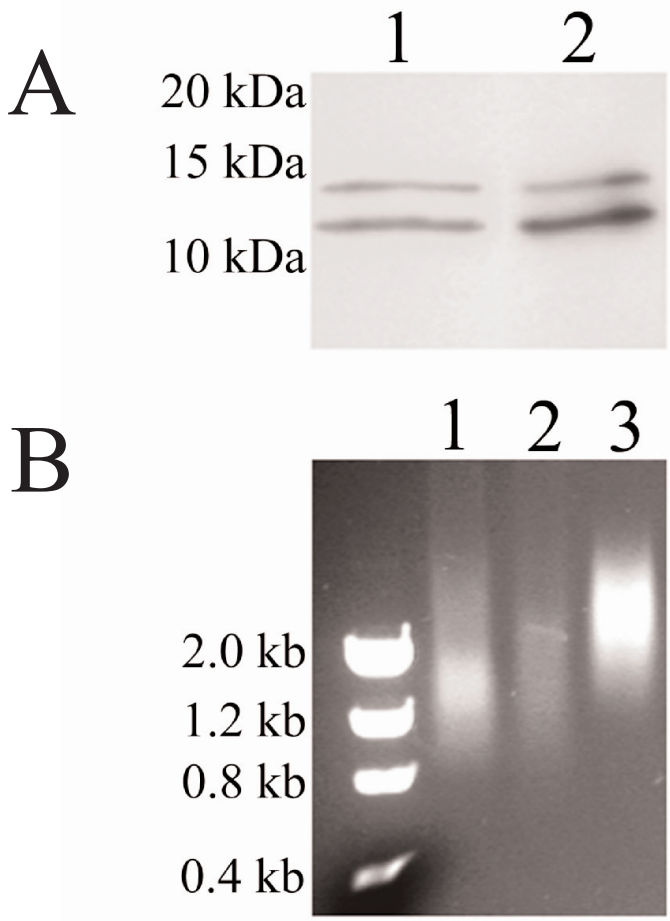

Figure 3. Histones and DNA are present in blocking reagents. **A.** Histones were identified in milk by SDS-PAGE and immunoblotting using anti-histone autoantibody KM-2, recognizing histone H2A and H4. Therefore, only two out of the four bands are visible (lane 1: 0.5 mg milk). Calf thymus histones were used as a marker (lane 2). Histones could not be detected in FCS and could barely be detected in BSA (data not shown). **B.** DNA was directly identified in 10 µl of 10% milk, 100% FCS and 25% BSA (lanes 1, 2 and 3, respectively).

blocking reagent on the reactivity can only be explained by the presence of nucleosomal material in the blocking reagent. Indeed, we directly identified histones in milk on immunoblots probed with anti-histone autoantibody KM-2, which recognizes both histone H2A and H4 (Fig. 3A). The amount of histones present in nonfat dry milk was estimated to be 1.5 µg/mg milk, by comparison of the intensity of the histone bands to a standard of calf thymus histones. We found no histones in FCS and a barely detectable amount in 99% pure BSA (data not shown). We also identified the presence of DNA in milk, FCS and BSA (Fig. 3B).We estimated this to be 1000 ng/ml in milk, 150 ng/ml in FCS and 70 ng/ml 99% in pure BSA. The isolated DNA from all three blocking reagents was sensitive to DNaseI treatment (data not shown).

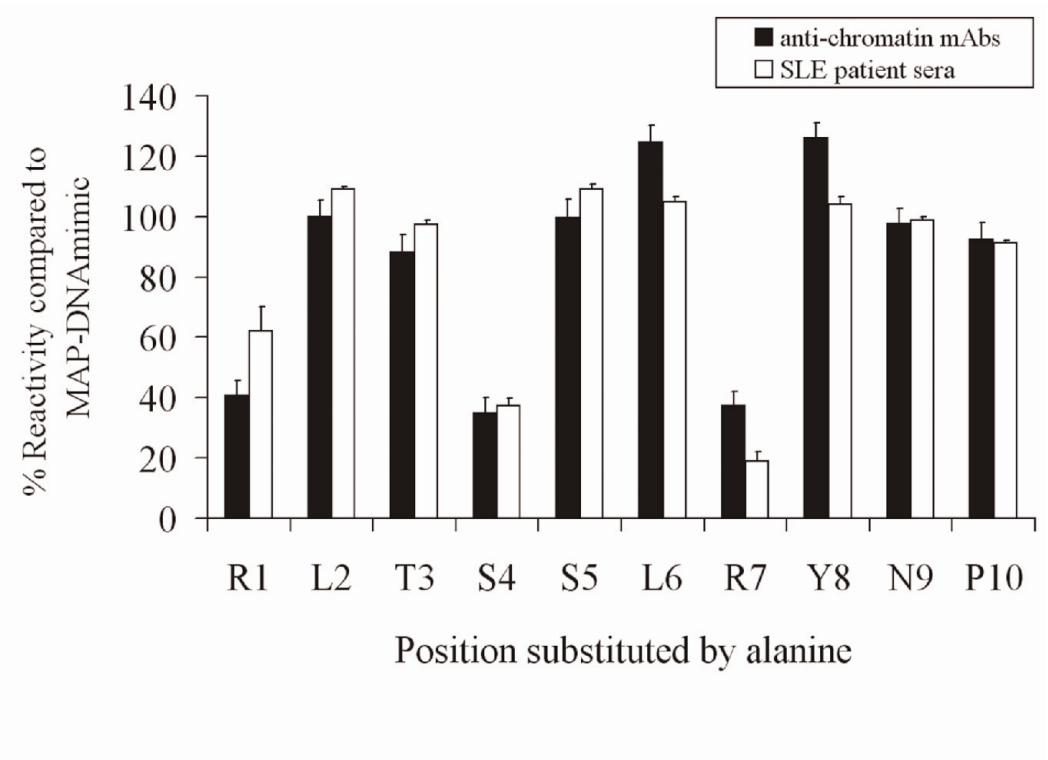

Figure 4. The motif within the MAP-DNAmimic was involved in binding to dsDNA as determined by alanine scanning. The effect of replacing each amino acid by alanine on binding is compared to the reactivity with the unsubstituted MAP-DNAmimic (=100%) as determined in a direct ELISA using milk as the blocking reagent containing DNA. The results are expressed as mean reactivity (±S.D.) of the positive anti-chromatin autoantibodies (i.e. #32, NZA2, LG10-1, PL2-3, #34, KM-2, #36 and #42) and three anti-DNA positive sera from SLE patients. The arginine residues at positions 1 and 7 and the serine residue at position 4 are critical for dsDNA binding.

Identification of the DNA-binding motif of the 'DNAmimic'

We performed an alanine scan to identify the motif in the 'MAP-DNAmimic' involved in the binding of DNA. MAP-peptides, which had one of the amino acids of the RLTSSLRYNP motif substituted by an alanine residue, were tested with the panel of anti-chromatin autoantibodies in a direct ELISA (Fig. 4). The reactivity for all the anti-chromatin autoantibodies was decreased by 40% or more compared to the unmodified MAP-DNAmimic, when the arginines on positions 1 and 7 or the serine on position 4 were substituted. The substitution of the other residues by an alanine had no effect on the binding of DNA as revealed by the binding of the anti-chromatin autoantibodies. Since the initial results with the MAP-DNAmimic were found with anti-DNA positive sera from SLE patients (89), we also performed the alanine scanning with some of the original SLE patient sera used for selection of the DNAmimic. These anti-DNA positive sera from SLE patients showed a similar decrease of binding with alanine substitutions in positions 1, 4 and 7 (Fig. 4).

Discussion

A mimotope for the nucleosome-specific antibody #32 was selected using a 10-mer random peptide phage display library. The motif NRKDWIERTL contains several positively and negatively charged amino acids, which might mimic, respectively the positively charged histones and the negatively charged DNA within the nucleosome. Surprisingly, besides antibody #32, a panel of other nucleosome-specific, anti-DNA and anti-histone antibodies also showed reactivity. The poly-reactivity of these anti-chromatin autoantibodies was not restricted to the mimotope we selected, but was also observed with a previously identified DNA mimotope (89) and a histone H3 peptide. We previously reported that the binding of DNA/histone complexes to anti-chromatin antibodies, generated during hybridoma cell culture, causes a similar poly-reactivity (94). Since this latter finding, we have extensively purified our anti-chromatin autoantibodies, until we cannot detect any complexed histones or dsDNA. The use of these noncomplexed purified anti-chromatin autoantibodies excluded the antibody preparation as a source for histones or dsDNA.

One of the explanations of the observed poly-reactivity may be the presence of DNA and histones in the blocking reagents used in the ELISA. Indeed, we were able to demonstrate that DNaseI treatment of the blocking reagents prevented the poly-reactivity of anti-chromatin autoantibodies, with MAP-MIMO#0, the MAP-DNAmimic and MAP-H3(21-37). Importantly, the reactivity of the nucleosome-specific antibody #32, used for the selection of MIMO#0, completely lost its reactivity with MAP-MIMO#0 in ELISA, when DNaseI treated milk was used as blocking reagent. DNaseI treatment of the blocking reagent resulted in complete degradation of the DNA (data not shown). Moreover, we found that dsDNA was able to bind directly to these peptides. The presence of DNA and/or histones in milk and bovine serum has been described previously (222, 223). In agreement with these results, we identified DNA and/or histones in bovine nonfat dry milk and FCS, and additionally also in BSA (purity 99%). Since we found no reactivity with any of the anti-chromatin autoantibodies after direct coating of the blocking reagents (data not shown), the effect of histones and dsDNA in the blocking reagents is only observed when a DNA- or histone-capturing molecule, i.e. the mimotope or histone peptide, is coated.

The identification of these DNA-binding peptides from the peptide phage display library and the blocking reagent-dependent reactivity in ELISA of the nucleosome-specific antibody, used for selection of MIMO#0, with the MAP-MIMO#0 peptide suggests that DNA or DNA/histone complexes were also present during the selection of phages. By their presence, this reagent-derived chromatin may mediate the binding of DNA- or histone-binding phages to the antibody used

for selection. Practically, it is almost impossible to determine which reagent has provided this chromatin bridge between the selected phage and the antibody used for selection. In addition to the blocking reagents, the chromatin-bridge may have originated from phages, bacteria or the yeast extract, which all contain DNA and/or histones by definition. The two mimotopes for two anti-DNA antibodies that have been reported by Sibille et al. (88) were selected in the absence of blocking reagents, but other sources of chromatin were still present (i.e. bacteria, phages, yeast extract). Furthermore, these mimotopes were not only recognized by the antibody used for selection, but also by the other antibody.

Although the selection of phages in the complete absence of chromatin seems impossible by definition, the characterization of mimotopes for anti-chromatin antibodies in ELISA should only be performed with blocking reagents devoid of DNA, histones or nucleosomes. However, to the best of our knowledge such blocking reagents are not available and should be developed. We identified the motif necessary for binding of dsDNA in the DNAmimic by performing an alanine scan. The arginines on positions 1 and 7 and the serine on position 4 of the DNAmimic were crucial for DNA binding. A motif containing two arginines, separated by four or five other amino acids, has been shown to be directly involved in the binding of DNA in several DNA-binding proteins (224). We did not perform an alanine scanning of the MIMO#0 peptide, but compared to the DNAmimic, it also contains the two arginines (positions 2 and 8) separated by five other amino acids. This motif, in combination with the asparagine on position 3 of MIMO#0, has been found in the DNA-binding domain of zinc finger proteins (225). Moreover, binding of proteins, containing this motif, to non-immunogenic DNA could help to induce an autoimmune response against DNA and other DNA-binding proteins such as histones (226). However, we did not observe any effect when we immunized normal and lupus-prone mice with the MIMO#0 peptide (data not shown). Binding of dsDNA to MAP-H3(21-37) might be expected, since this peptide is part of the N-terminal tail of histone H3. Selection of mimotopes for anti-DNA and nucleosome-specific antibodies does not always have to result in the identification of DNA-binding peptides since we could not show binding of labeled DNA to the mimotope peptide D/E-W-D/E-YS/G (87) (not shown), but the possibility remains that this particular peptide specifically binds histones and/or nucleosomes. In conclusion, our results show that the presence of reagent-derived nucleosomal material is a serious problem when anti-chromatin antibodies are used for both screening of random peptide phage display libraries and the subsequent characterization of epitope/mimotope peptides in ELISA. Preliminary results also indicate that the reactivity of SLE patient sera with DNA, histones and nucleosomes greatly depends on the blocking reagent used (data not shown). The influence of chromatin present in the blocking reagent on the reactivity of anti-chromatin autoantibodies may also

play a role in commercially available tests that rely on the use of conventional blocking reagents. Efforts should be made to develop blocking reagents totally devoid of chromatin. Since such a blocking reagent does not yet exist, we recommend at this stage that FCS be used for general serological testing when anti-DNA responses are measured, and that DNaseI-treated milk be used when anti-histone reactivity is analyzed.

Acknowledgements

We would like to thank Dr. M. Scharff (Albert Einstein College of Medicine, New York) for providing the L100 random peptide phage library and Dr. J.P. Briand (CNRS, Strasbourg) for synthesizing peptides. This work was supported by the Dutch Kidney Foundation grant C99.1826.

Chapter 7.

A lupus-derived anti-histone autoantibody specific for histone variant H3.1

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Adapted from Mechanisms in Development 2005; in press

Abstract

Anti-nucleosome autoantibodies are most frequently found in patients with the autoimmune disease systemic lupus erythematosus (SLE). Nucleosomes are the basic structure of chromatin and consist of 146 base pairs of dsDNA wrapped around an octamer of the core histones H2A, H2B, H3 and H4. Several epitopes targeted in lupus by T cells and autoantibodies have been identified in the core histones. Here we mapped the epitope of a monoclonal anti-histone histone autoantibody, mAb #34, derived from the MRL/lpr lupus mouse, by screening several random peptide phage display libraries. We identified the common motif S/G-A-P-A-S/T-G, which corresponds to amino acid residues 28-33 in the N-terminal region of histone H3.1. Within this N-terminal region the single amino acid difference between the histone H3 variants H3.1 and H3.3 is located, i.e. at position 31 a serine residue in H3.1 versus an alanine residue in H3.3, respectively. We demonstrated that #34 was specific for H3.1 both in direct and inhibition ELISA. Compared to the unmodified H3.1 peptide, we found in ELISA a less pronounced inhibitory effect of the H3.3 peptide and H3.1 peptides phosphorylated at S28 and/or acetylated at K18 and K23. A H3.1 peptide di-methylated at K27 showed an inhibitory effect comparable to the unmodified H3.1 peptide. In conclusion, we identified #34 as an anti-histone autoantibody specific for (unmodified) histone H3.1.

Systemic lupus erythematosus (SLE) is an autoimmune disease driven by autoantigens, of which the nucleosome has been identified as a major autoantigen (13). Nucleosomes are the basic structure of chromatin, which enables the compact folding of DNA in the nucleus. Each nucleosome consists of 146 base pairs of double stranded (ds) DNA wrapped around an octamer of the core histones H2A, H2B, H3 and H4. For a long time, anti-DNA autoantibodies have been regarded as the serological marker for SLE, however DNA itself is not immunogenic. When DNA is bound to histones or microbial proteins, it can induce an anti-nucleosome and anti-DNA response (9, 10). Indeed, nucleosome- and histone-specific T and B cells have been identified (54-59, 214, 227-229). By using overlapping histone H3 peptides B- and T cells epitopes have been identified in histone H3, i.e. amino acids 53-85, 85-119 and 111-130 (55, 58, 227). In addition, reactivity of sera from lupus mice and patients with the N-terminal tail of histone H3 has been found (212, 230). However, the exact amino acid sequences recognized by some anti-histone autoantibodies are unknown. Previously, Smeenk and co-workers characterized several monoclonal autoantibodies (mAb) derived form lupus mice (81), including anti-histone mAb #34 derived from the MRL/lpr lupus mouse. We already demonstrated that this antibody showed a high reactivity with nucleosomes

and histones, and a low reactivity with a histone H3 peptide, i.e. amino acids 18-32 (94, 221).

In this study, we identified the exact epitope of mAb #34 by screening several random peptide phage display libraries. The epitope of mAb #34 was found in the N-terminal tail of histone H3, as S/G-A-P-A-S/T-G (amino acids 28-33). Using synthetic peptides we could demonstrate that mAb #34 is specific for the histone variant H3.1 and not for the H3.3 variant, which differs in the N-terminal region of H3 at amino acid position 31 from the H3.1 variant (A->S). Furthermore, we demonstrated that mAb #34 preferentially binds to the unmodified H3.1 peptide (amino acids 18-37) compared to the corresponding peptides acetylated and/or phosphorylated at K18, K23 and/or S28, respectively. However, mAb #34 showed a comparable reactivity with a H3 peptide that was di-methylated at K27. We conclude that mAb #34 is a histone-specific autoantibody that primarily recognizes (unmodified) H3.1.

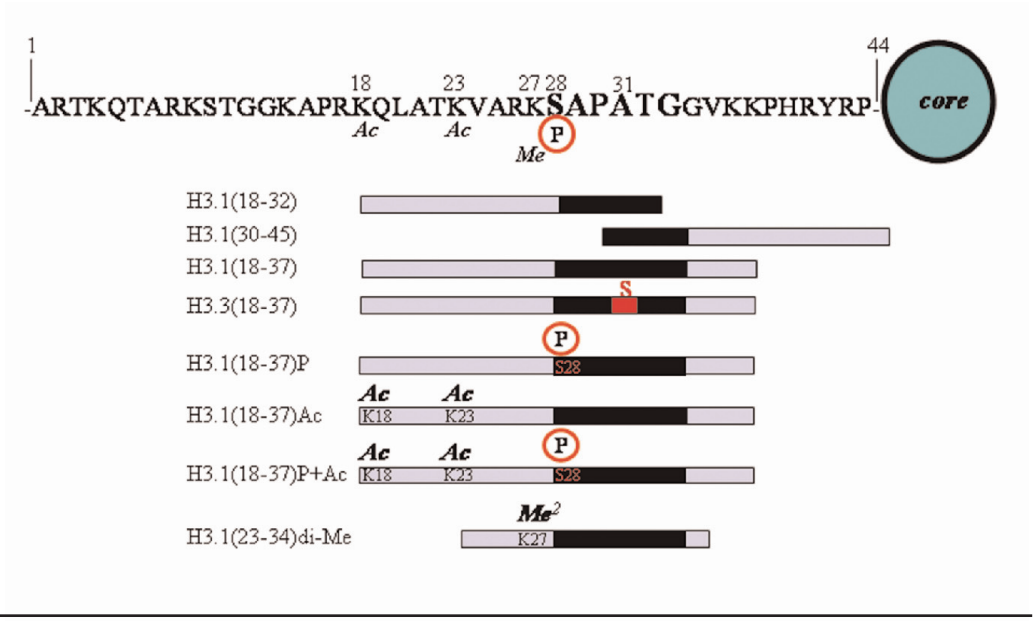

Figure 1. Histone H3 peptides tested in ELISA. The N-terminal tail of histone H3 (amino acid 1-44) is depicted, which can be modified by either acetylation of lysine, methylation of lysine or arginine and phosphorylation of serine or threonine residues. We tested H3 peptides with the H3.1 or H3.3 sequence, or modified residues, i.e. acetylated at K18 and K23 and/or phosphorylated at S28. The black shaded region corresponds to the identified epitope (28-33) for mAb #34.

Materials & Methods

Random peptide phage display

Antibody #34 is a monoclonal anti-histone autoantibody, derived from the (NZBxNZW)F1 lupus mouse model, and was purified as described previously (94). The epitope recognized by antibody #34 was determined by screening a 10-mer (kindly provided by Dr. C. Putterman), a 12-mer (New England Biolabs) and a 15-mer (kindly provided by dr. G. Smith) random peptide phage display library with mAb #34, as described previously (97, 217).

Direct and inhibition ELISA

The reactivity of mAb #34 was analyzed in a direct and an inhibition ELISA with the H3(18-32) and H3(30-45) peptides, and several H3(18-37) peptides, containing either the H3.1 or H3.3 sequence, or one or more modified residues (i.e. acetylated and/or phosphorylated at K18, K23 and/or S28, respectively), and the H3(23-34) peptide di-methylated at K27 (Abcam, Cambridge, U.K.). All peptides were synthesized in the peptide synthesis facility of the CNRS in Strasbourg, except for the H3.3(18-37) peptide (Eurosequence, Groningen, The Netherlands). For direct ELISA, Maxisorb® 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 µl of peptide at a concentration of 2 µM in 50 mM carbonate buffer pH 9.6 overnight at 4°C. The wells were washed with PBS/0.05% (v/v) Tween-20 (PBS-T) and blocked with 150 µl 1% BSA for 2 hours at room temperature. After washing with PBS-T, 100 µl of mAb #34 starting at a concentration 10 µg/ml were added in PBS-T and incubated for 2 hours at room temperature. After washing with PBS-T, 100 µl horseradish peroxidase-conjugated goat anti-mouse Ig(H+L) (Southern Biotechnology Associates, Birmingham, U.S.A.) diluted 1:7500 in PBS-T were added and incubated for 1 hour at room temperature. The plates were washed again with PBS-T and developed for 15 minutes using 100 µl 3,5,3',5'-tetramethylbenzidine (TMB) (SFRI Diagnostics, Saint Jean d'Ilac, France). The reaction was stopped by the addition of 100 µl 2 M H_2SO_4 and the optical density at 450 nm was measured in a microplate reader (Biorad, Veenendaal, The Netherlands).

For inhibition studies, 0.5 µg/ml of mAb #34 diluted in PBS-T was pre-incubated in a 96-wells plate (Greiner, Alphen a/d Rijn, The Netherlands) with a series of dilutions of a competitor peptide, and added to a Maxisorb® 96 plate coated with (unmodified) H3.1(18-37) peptide.

Results

To map the epitope of mAb #34, we first screened a 10-mer random peptide phage display library. This resulted into the consensus motif: S/G-A-P-A-S/T-G, which was deduced from clones obtained after 3 rounds of selection with a decreasing concentration of mAb #34 (i.e. 1 µM, 10 nM and 100 pM) (Table I). We could deduce the same motif from clones selected after screening a 12-mer and a 15-mer library (not shown). The sequence of the selected motif corresponded to amino

Table I. Motif deduced from phage clones of the L100 10-mer library selected by mAb #34.

	Sequence[1]																			
H3(22-40):	S	K	A	A	R	K	S	A	P	A	T	G	G	V	K	K	P	H	R	
				g	s	g	g	A	P	A	T	R	E	R	H	L	Q	g	a	p	s
	g	s	g	g	V	F	T	A	S	A	P	A	T	R	g	a	p	s			
				g	s	g	g	A	P	A	S	W	T	F	A	R	S	g	a	p	s
	g	s	g	g	N	R	W	S	G	G	A	P	A	T	g	a	p	s			
		g	s	g	g	P	G	R	P	A	P	A	T	R	W	g	a	p	s		
				g	s	g	g	Q	A	P	A	T	W	V	R	E	W	g	a	p	s
					g	s	g	g	A	P	A	T	G	W	I	W	K	S	g	a	p	s
				g	s	g	g	E	A	P	R	G	A	W	Y	R	Q	g	a	p	s
					g	s	g	g	A	P	A	T	R	V	R	A	I	V	g	a	p	s
	g	s	g	g	S	G	M	M	S	A	P	A	T	A	R	g	a	p	s		
				g	s	g	g	S	A	P	A	T	R	Q	G	K	W	g	a	p	s
					g	s	g	g	A	P	A	S	W	T	F	G	R	S	g	a	p	s
		g	s	g	g	Q	I	R	S	A	P	A	T	G	A	g	a	p	s		
Motif:							S/G	A	P	A	S/T	G									

[1] Amino acid sequence of the pIII fusion protein flanking the random amino acid residues are designated in lower case.

acid residues 28-33 of the N-terminal region of histone H3.1. Previously, we observed a low reactivity of mAb #34 with a histone H3(18-32) peptide (221). Here we found that mAb #34 had a high reactivity with the H3(18-37) peptide, while no reactivity with the H3(1-21), H3(30-45) and H3(18-32) peptides could be observed, although this latter peptide contained almost the entire identified S/G-A-P-A-S/T-G sequence (data not shown). Since mAb #34 shows a high reactivity with nucleosomes (94), which initially led to the assumption that it was a nucleosome-specific autoantibody, we tested the effect of adding DNA to a coated H3(18-37) peptide. However, the binding of mAb #34 to H3(18-37) was not affected by the addition of DNA (not shown). These results suggested that mAb #34 is actually a histone-specific antibody.

Within the identified epitope (amino acids 28-33) for mAb #34 resides the single N-terminal amino acid difference between the H3 variants H3.1 and H3.3, i.e. at position 31 an alanine versus a serine residue, respectively (Fig. 1). We demonstrated in direct ELISA that mAb #34 exclusively binds to the histone

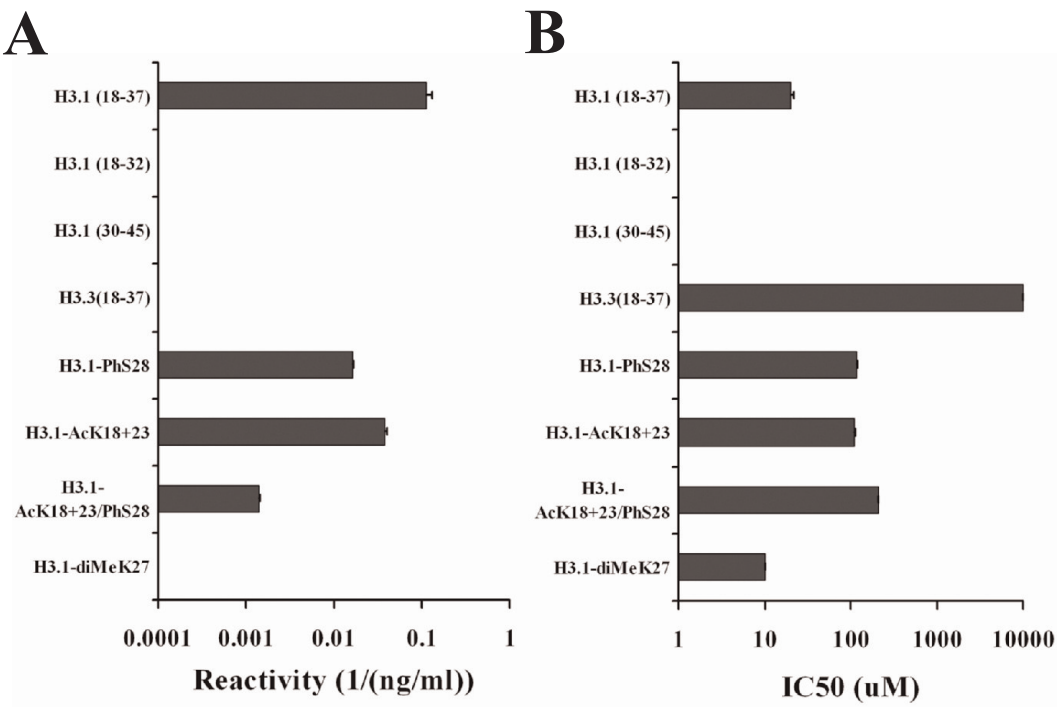

Figure 2. Reactivity of mAb #34 with (modified) histone H3.1 and H3.3 peptides in ELISA. **A.** Reactivity of #34 was tested with directly coated peptides. The reactivity is calculated as the reciprocal of the concentration of #34 that yields an absorbance of 0.5 at 450 nm. **B.** Inhibition of the binding of #34 to unmodified H3.1(18-37) by (modified) H3.1 and H3.3 peptides. The inhibitory concentration (IC) of the peptide that yields a 50% decrease of reactivity of #34 with H3(18-37) is given as the IC50.

H3.1(18-37) peptide and not to the H3.3(18-37) peptide (Fig. 2A). Moreover, the H3.3 peptide was not able to inhibit the binding of mAb #34 to the H3.1 peptide in an inhibition ELISA (Fig. 2B).

Modifications of amino acid residues close to or in the S/G-A-P-A-S/T-G -motif have been described to occur *in vivo* (see Fig. 1) (14). We tested in a direct and an inhibition ELISA the influence of some of these modifications on the reactivity of mAb #34. H3 peptides acetylated at lysine 18 and 23 and/or phosphorylated at serine 28, showed a decreased binding of mAb #34 and an about 5-fold lower inhibitory effect compared to the unmodified H3.1 peptide (Fig. 2, A and B). However, this effect was much less pronounced when compared to the H3.3 peptide. We were not able to demonstrate direct binding of #34 to a H3.1 (23-34) peptide di-methylated at lysine 27, but this could be explained by inefficient coating of this shorter peptide. However, in the inhibition ELISA the di-methylated H3(23-34) peptide showed an inhibition comparable to the unmodified H3.1 peptide (Fig. 2, A and B).

Discussion

In this study, we have identified the epitope of a monoclonal anti-histone autoantibody, mAb #34, in the N-terminal tail of histone H3, i.e. amino acids 28-33. In the past, we have shown that after extensive purification, mAb #34 showed a high reactivity with nucleosomes, a lower reactivity with histones and a very low reactivity with a histone H3(18-32) peptide (94, 221). Since for the H3(18-37) peptide a much higher reactivity was observed than for the H3(18-32) peptide, and the di-methylated H3(23-34) peptide showed an inhibition comparable to the unmodified H3(18-37) peptide, we conclude here that mAb #34 requires amino acid residues 28-33 for optimal binding.

Four different variants of histone H3 have been reported: H3.1, H3.2 and H3.3, which have a similar amino acid sequence, and the completely different centromeric histone H3 variant, CENPA (231). Recently, H3.3 has been associated with transcriptionally active sites in chromatin, while H3.1 is tightly coupled to DNA replication and transciptionally silent chromatin regions (232, 233). The full sequence of H3.1 and H3.3 differs only at four amino acid residues, the three different residues in the globular domain (i.e. position 87, 89 and 90) which are crucial for the distinctive function of the H3.3 variant (232), and a single amino acid difference in the N-terminal tail, for which no clear role has been designated yet. The N-terminal amino acid difference between the histone H3 variants H3.1 and H3.3, i.e. at position 31 an alanine residue in H3.1 and a serine residue in H3.3, resides in the epitope we identified for mAb #34. We showed that mAb #34 is specific for histone H3.1, since a peptide corresponding to the N-terminal H3.3 sequence was not recognized by mAb #34 and could not inhibit the binding of the antibody to the H3.1 peptide. Additionally, we tested several modifications, i.e. acetylation, phosphorylation and methylation, which have been described in or close to the identified epitope. The phosphorylation of S28 has been associated with apoptosis, and could be an apoptosis-induced modification targeted by autoantibodies in SLE (21, 25, 234, 235). We found that acetylation and/or phosphorylation resulted in a decreased binding of mAb #34, while methylation had a minor effect on the binding. However, the inhibitory effect of these modifications was much less pronounced compared to the inhibitory effect of the single amino acid substitution at position 31 in the H3.3 peptide. Since antibodies specific for histone H3 variants are rare, the particular specificity of mAb #34 led to the successful application in a study concerning the presence of histone H3 variants in the early mouse zygote (98). In this study asymmetry for histone H3 variants in the pre-S-phase zygote was demonstrated for the paternal and maternal derived nucleus, since staining with mAb #34 showed that H3.1 is virtually absent from the paternal chromatin, while localization of the histone H3.3/H4 assembly factor HIRA with the paternal

chromatin indicated the presence of histone H3.3. Moreover, double staining of early zygotes with mAb #34 and antibodies specific for modifications in or nearby the epitope of #34 (i.e. acetylation of K18 or K23, mono-/di-/tri-methylation of K27 and phosphorylation of S28) never revealed a co-localization between mAb #34 and the mAb specific for H3 phosphorylated at S28, while staining with antibodies specific for the other modified forms of H3 sometimes revealed a partial co-localization with mAb #34. Combining all results, we conclude that antibody #34 primarily recognizes (unmodified) histone H3.1.

Chapter 8.

The binding of lupus-derived autoantibodies to the C-terminal peptide (83-119) of the major SmD1 autoantigen can be mediated by DNA and nucleosomes

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Jo Berden & Johan van der Vlag
Submitted

Abstract

Objectives: To evaluate the binding of lupus-derived autoantibodies, dsDNA and nucleosomes to the positively charged SmD1(residues 83-119) peptide.
Methods: Binding of lupus-derived monoclonal antibodies, SLE patients' sera, dsDNA and nucleosomes to the SmD1(83-119) peptide was determined using different ELISA methods.
Results: Anti-dsDNA antibodies and SLE patients' sera reacted with the SmD1(83-119) peptide, while DNaseI treatment of the applied reagents led to a decreased reactivity. Purified dsDNA and nucleosomes bound to the peptide.
Conclusions: The SmD1(83-119) peptide is able to bind dsDNA and nucleosomes, and dsDNA/nucleosomes in applied reagents can lead to an apparent reactivity of anti-dsDNA, anti-histone or nucleosome-specific antibodies with the SmD1(83-119) peptide.

In systemic lupus erythematosus (SLE) a variety of autoantibodies against nuclear components such as nucleosomes (histones and dsDNA) and spliceosomal proteins (e.g. SSA/Ro, SSB/La, snRNP, Sm and hnRNP antibodies) exist. The autoantibody production appears to be antigen driven and T cell dependent. Recently, the glycine-arginine-rich C-terminal part (residues 83-119) of the spliceosomal SmD1 protein has been identified as a major autoepitope targeted by sera from 36-70% of SLE patients (236, 237). The reactivity with the SmD1(83-119) peptide correlates to the anti-dsDNA reactivity and disease activity (236, 237, 243). Furthermore, SmD1(83-119)-reactive T cells can stimulate the production of pathogenic anti-dsDNA antibodies and administration of the SmD1(83-119) peptide to NZB/NZW lupus mice strongly accelerates the progression of the disease (238, 239).

Anti-Sm reactivity has been included as one of the classification criteria for SLE by the American College of Rheumatology and at present the anti-SmD1(83-119) reactivity in ELISA is routinely measured. The binding of dsDNA to the positively charged C-terminal tail of the SmD1 protein may play a role in the development of the anti-chromatin immune response in SLE (226, 239), but may also interfere with ELISA measurements (97). Therefore, we evaluated the binding of lupus-derived monoclonal anti-dsDNA, anti-histone, nucleosome-specific antibodies (NSA), SLE patients' sera, dsDNA and nucleosomes to the SmD1(83-119) peptide using different ELISA methods.

Materials & Methods

Monoclonal antibodies and patient sera

Monoclonal anti-dsDNA (#36, #42, #56), anti-histone (KM-2 and #34) or NSA (PL2-3, #32 and LG10-1) were derived from (NZBxNZW)F1 lupus mice, except PL2-3 (MRL/+ lupus mice) and KM-2 and LG10-1 (MRL/lpr lupus mice) (97). Following DNaseI treatment of the hybridoma culture supernatants, the monoclonal antibodies were thoroughly purified as described (82). WT-32 (anti-CD3 antibody) was used as a control. A rabbit anti-SmD1(83-119) peptide antiserum was described (237). SLE patients' sera were collected at the Radboud University Nijmegen Medical Centre and the Charité University Hospital, and approved by the local ethical committees.

Anti-SmD1(83-119) peptide and anti-dsDNA ELISA

Anti-SmD1(83-119) reactivity was determined in ELISA essentially as described (237). Coated SmD1(83-119) peptide was blocked with PBS/0.05% (v/v) Tween-20 (PBS-T) with 1% blocking reagent, i.e. non-fat dry milk (Bio-Rad Laboratories, Veenendaal, The Netherlands), casein (Sigma, Zwijndrecht, The Netherlands) or BSA for 2 hours at room temperature (RT). Wells were incubated with serially diluted (in PBS-T) monoclonal antibody, starting with a concentration of 5 μg/ml, or 1:100 diluted SLE patients' serum for 2 hours at RT. For detection horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:30,000 or goat anti-human IgG diluted 1:15,000 (Southern Biotechnology Associates, Birmingham, U.S.A.) in PBS-T was used. Anti-dsDNA ELISA and DNaseI treatment of reagents were performed as described (97).

Binding of dsDNA, biotin-labeled dsDNA or nucleosomes to the SmD1 (89-119) peptide

dsDNA was labeled with biotin according to the manufacturer's instructions (Roche). Coated SmD1(83-119) peptide was blocked with DNaseI-treated milk or casein, or BSA (1%) and incubated with serially diluted (in PBS-T) dsDNA (Roche), biotin-labeled dsDNA or nucleosomes (quantity based on the absorbance at 260 nm) for 30 minutes at RT. The remainder of the ELISA was performed as described (97).

Results

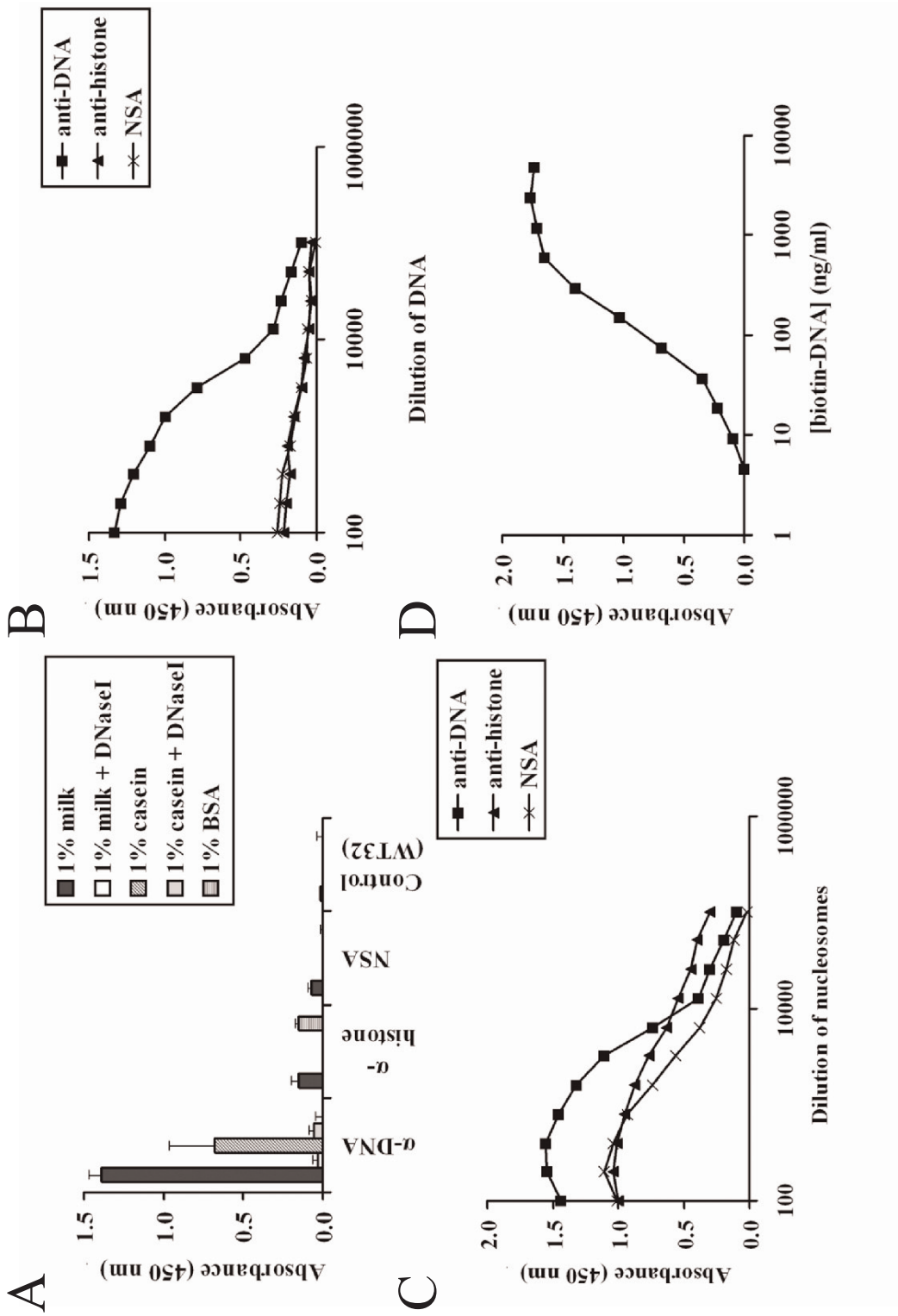
Apparent reactivity of anti-dsDNA antibodies and binding of dsDNA and nucleosomes to the SmD1(83-119) peptide

The reactivity of lupus-derived monoclonal anti-dsDNA, anti-histone and NSA with the SmD1(83-119) peptide was determined in ELISA. Using milk or casein as blocking reagent, all anti-dsDNA antibodies showed a high reactivity with the peptide, while the anti-histone and NSA hardly reacted (Fig. 1A). With BSA as blocking reagent only a very low reactivity of anti-histone antibodies could be observed (Fig. 1A). Recently, it appeared that the application of different blocking reagents led to different reactivities of SLE patients' sera with the SmD1(83-119) peptide (240), while we showed that the presence of nucleosomal material in common blocking reagents interfered with the reactivity of anti-chromatin autoantibodies in ELISA (97). Therefore, we also tested the effect of DNaseI treatment of the applied blocking reagent, i.e. milk and casein, which indeed abolished the reactivity of all monoclonal anti-dsDNA antibodies with the SmD1(83-119) peptide (Fig. 1A). The addition of purified dsDNA (Fig. 1B) or nucleosomes (Fig. 1C) to the coated SmD1(83-119) peptide not only restored the reactivity of anti-dsDNA antibodies, but also revealed reactivity of NSA and anti-histone antibodies with the peptide (Fig. 1, B and C). We could also demonstrate direct binding of biotin-labelled dsDNA to the SmD1(83-119) peptide (Fig. 1D).

DNaseI treatment of blocking reagent and/or serum abolishes anti-SmD1(83-119) reactivity

We now questioned whether the dsDNA and nucleosomes present in blocking reagents could also interfere with the reactivity of SLE patients' sera with the SmD1(83-119) peptide. Indeed, DNaseI treatment of the blocking reagent, i.e.

Figure 1. (next page) Binding of lupus-derived monoclonal anti-dsDNA, anti-histone, or nucleosome-specific antibodies (NSA) with the SmD1(83-119) peptide. The reactivity of the antibodies was determined in ELISA with milk or casein, either or not treated with DNaseI, or BSA (1%) as blocking reagent. WT-3 served as a control antibody (**A**). Reactivity of anti-dsDNA, anti-histone and NSA with the SmD1(83-119) peptide, using BSA (1%) as blocking reagent, after addition of dsDNA, starting at 100 ng/ml (**B**), or nucleosomes, starting at 74 ng/ml (**C**). Binding of biotin-labelled DNA to the SmD1(83-119) peptide determined in ELISA with DNaseI-treated milk (1%) as blocking reagent (D). All results are expressed as absorbance values at 450 nm. The mean (±SD) of six experiments is shown.


A
Absorbance (450 nm)
1.5
1.0
0.5
0.0
1% milk
1% milk + DNaseI
1% casein
1% casein + DNaseI
1% BSA
α-DNA
α-histone
NSA
Control (WT32)
B
Absorbance (450 nm)
anti-DNA
anti-histone
NSA
100
10000
1000000
Dilution of DNA
C
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0.0
anti-DNA
anti-histone
NSA
100
10000
1000000
Dilution of nucleosomes
D
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0.0
1
10
100
1000
10000
[biotin-DNA] (ng/ml)

1% milk, resulted on average in a 90% decreased reactivity of 6 (designated group I) out of 8 tested sera, while two sera (designated group II) retained about 60% of their original reactivity. The reactivity of the rabbit anti-SmD1(83-119) peptide antiserum was not affected (Fig. 2). DNaseI treatment of sera resulted on average in a 20% decreased reactivity, while the reactivity of the rabbit serum was not altered (Fig. 2). A combination of DNaseI treatment of both the blocking reagent and the sera almost completely abolished the reactivity of 6 out of 8 sera (group I), while two sera (group II) retained about 60% of their original reactivity (Fig. 2). All 8 sera showed a high reactivity with dsDNA, while the rabbit serum showed no reactivity (data not shown). In contrast to the anti-SmD1(83-119) reactivity, DNaseI treatment of reagents did not change the reactivity of the sera with dsDNA (data not shown).

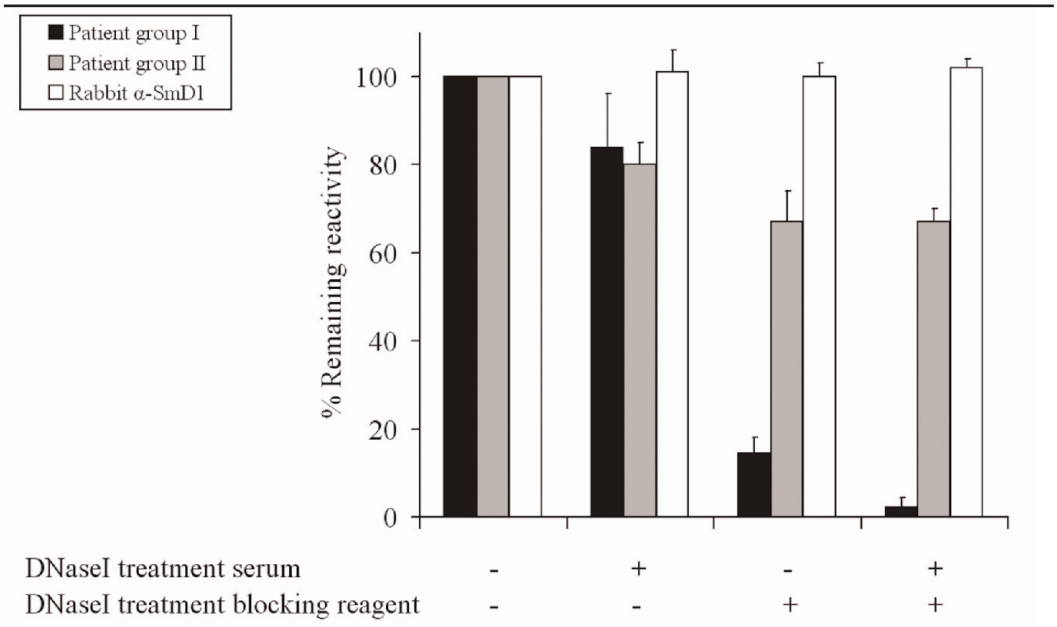


Figure 2. The reactivity of SLE patients' sera with the SmD1(83-119) peptide and the effect of DNaseI treatment. The reactivity of the sera from 8 SLE patients, either or not treated with DNaseI, was determined in ELISA with milk (1%), either or not treated with DnaseI, as blocking reagent. A rabbit anti-SmD1(83-119) peptide antiserum served as a control. Results are expressed relative to the reactivity obtained with untreated blocking reagents and sera, which is set to 100%. The mean (±SD) of six experiments is shown.

Discussion

We demonstrate that lupus-derived monoclonal anti-dsDNA antibodies can bind to the spliceosomal SmD1(83-119) peptide when milk or casein were used as blocking reagents, while anti-histone and NSA hardly reacted with the SmD1(83-119) peptide. Apparently dsDNA/nucleosomes, which were present in the applied blocking reagents, mediated the SmD1(83-119) reactivity of anti-dsDNA antibodies, since DNaseI treatment abolished the reactivity. In line with these findings we recently showed that nucleosomal material present in blocking reagents could interfere with the binding of lupus-derived anti-chromatin antibodies in ELISA (97). The SmD1(83-119) peptide was able to bind purified dsDNA and nucleosomes, which in addition to the reactivity of anti-dsDNA antibodies, revealed reactivity of anti-histone and NSA. Taken into account the presence of 16 (out of 37) positively charged residues in the SmD1(83-119) peptide and the negative charges abundantly present in dsDNA and nucleosomes, the binding of dsDNA and nucleosomes to the peptide was not unexpected and previously it has been shown that ss/dsDNA can bind to Sm proteins (245). Also the reactivity of 6/8 SLE patients' sera with the SmD1(83-119) peptide was completely abolished after DNaseI treatment of the reagents, while 2 sera retained their reactivity. These data may explain that the use of different ELISA methods may lead to great differences (36-70%) in the prevalence of anti-SmD1(83-119) reactivity in SLE patients (236, 237). Using other methods the binding of anti-dsDNA antibodies to the intact SmD1 protein and binding of anti-SmD1 antibodies to ss/dsDNA has also been demonstrated (241-243, 245, 246, 289). The in vivo formation of dsDNA/SmD1 complexes and other dsDNA/protein complexes may play an important role in the development of the anti-dsDNA response in SLE (226, 239) and anti-SmD antibodies mainly target the positively charged structures within spliceosomal proteins (247). Most importantly, it has been demonstrated that SmD1(83-119)-specific T cells provide help for the production of anti-dsDNA antibodies (239). Whether SmD1(83-119)-specific T cells also provide help for anti-histone or NSA remains to be investigated, but our present findings do point to the formation of nucleosome/SmD1(83-119) complexes.

In conclusion, the measurement of reactivity of sera with the SmD1(83-119) peptide in ELISA can lead to overestimation of the anti-SmD1 reactivity, since anti-dsDNA antibodies can also react. Therefore, to distinguish anti-dsDNA reactivity from genuine anti-SmD1(83-119) reactivity in ELISA, we recommend DNaseI treatment of all reagents and/or to use BSA as blocking reagent. Nevertheless, recognition of the dsDNA/SmD1(83-119) complex by anti-dsDNA antibodies, and of the nucleosome/ SmD1(83-119) complex by anti-dsDNA, anti-histone and NSA, is a novel finding, which may play a role in the development of the anti-chromatin immune response in SLE.

Chapter 9.

Nucleosomes and anti-nucleosome autoantibodies as mediators of glomerular pathology in systemic lupus erythematosus

Jürgen Dieker, Johan van der Vlag & Jo Berden

In: J. Kalden & M. Herrmann, editors. Apoptosis and Autoimmunity: From Mechanisms to Treatments. 1st ed; Weinheim: Wiley Europe; 2003. pp319-342

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the formation of autoantibodies directed against various autoantigens. These autoantibodies are often specific for antigens located in the nucleus, such as nucleosomes, histones and double stranded (ds) DNA, but also RNA-associated proteins, like SS-A, SS-B and Sm. Historically, antibodies against dsDNA are considered as a hallmark of the disease. Antibodies specific for dsDNA can be detected in about 70% of SLE patients and in about 96% of SLE patients with active disease (247). Increasing anti-DNA antibody levels also precede exacerbations of the disease (100). First, polyclonal B cell activation was regarded as the mechanism behind the formation of anti-DNA antibodies. Later, it became clear, however, that SLE is a T cell-dependent and autoantigen-driven autoimmune disease. Since naked DNA is a poor immunogen, the nucleosome, the basic structure of chromatin that consists of DNA and proteins, has been proposed as the major autoantigen (11). The significance of the nucleosome as a major autoantigen in SLE has been further substantiated by the identification of nucleosome-specific T helper cells (60) and the high prevalence of anti-nucleosome autoantibodies (12). In addition, nucleosomes have been detected in the circulation of SLE patients (49) and lupus mice (51). During apoptosis nucleosomes are clustered in apoptotic blebs at the surface of apoptotic cells (36). When the removal of apoptotic cells is impaired, nucleosomes may be released in the circulation due to the instability of these cells. Indeed, there is increasing evidence that apoptosis is disturbed in SLE (102). Apoptosis-induced modifications of autoantigens targeted in SLE may make them more immunogenic (152). This becomes particularly relevant if the removal of apoptotic cells is insufficient.

Often no distinction is made between anti-DNA and anti-nucleosome antibodies, which is confusing. Anti-DNA and anti-histone antibodies are specific for respectively DNA and histones, but also recognize nucleosomes. Anti-nucleosome antibodies are defined by their much higher specificity/reactivity towards the complete nucleosome compared to isolated histones or naked DNA. The total group of antibodies reacting with nucleosomes, histones and DNA are referred to as anti-chromatin antibodies. The prevalence of anti-nucleosome antibodies is even higher than that of anti-DNA antibodies. These anti-nucleosome antibodies are not exclusively found in SLE, but also in scleroderma and mixed connective tissue disease (68), although this latter finding was not confirmed in another study (70). Anti-nucleosome antibodies are detected before anti-DNA antibodies are present in the circulation, which indicates that they most likely are responsible for the subsequent induction of both the anti-DNA and the anti-histone antibody responses via antigen spreading (64).

Apart from their role as autoantigen, nucleosomes also play a role in mediating tissue lesions, especially glomerulonephritis (99). Nephritis is one of the most serious manifestations of SLE and develops in 40-50% of the SLE patients. Granular deposits of immunoglobulins and complement factors can be found in glomeruli in renal biopsies of SLE patients, which suggests an immune complex-mediated pathogenesis. Renal manifestations of the disease are often preceded by a rise in the level of anti-chromatin antibodies in the circulation, while these antibodies are also detected in immune deposits in the glomerulus (248). Apparently, anti-chromatin antibodies play an important role in the development of renal disease during SLE. However, the mechanism that explains the induction of nephritis by antichromatin is still under debate. Until now two different models have been formulated. The first model explains the deposition of anti-chromatin antibodies in the kidney by direct interaction of these antibodies with molecules present in the glomerular basement membrane (GBM). The second, in our opinion more favorable model, attributes the glomerular pathology to nucleosome-mediated deposition of immune complexes either formed in situ or originating from circulating immune complexes. Both models will be discussed in more detail in the next sections.

Reactivity of anti-chromatin antibodies with glomerular components

The causal relationship between autoantibody formation and nephritis in SLE has intrigued many investigators. A variety of autoantibody specificities has been assigned a pathogenic role in the development of lupus nephritis. Antibodies specific for known components of the GBM are not commonly found in SLE, but most sera are reactive towards the GBM, which led to the idea of cross-reactive recognition of glomerular components (249). In particular, the observation that murine and human monoclonal antibodies specific for dsDNA showed a cross-reactivity to several proteins, has been the basis to explain the binding of autoantibodies to glomerular components, especially in the GBM (250). This cross-reactivity has even been used to distinguish pathogenic anti-DNA antibodies in SLE sera from nonpathogenic anti-DNA antibodies present in normal sera (251). The cross-reactivity of anti-DNA autoantibodies has been described for various biomolecules, such as membrane structures like cardiolipin (252-255) and phosphocholin (256), ribosomal protein P1 (257), A and D snRNP (241), nuclear proteins like nuclear envelope proteins (258) and the transcription factor EF-2 (259).

The various *in vitro* and *in vivo* studies showing reactivity of anti-DNA antibodies with glomerular components or the GBM are listed in Tables I and II. Immunoglobulins in serum from lupus mice and patients show reactivity with

isolated glomeruli (260), which could be inhibited by the addition of DNA, but not by DNaseI treatment of the glomeruli. This finding may implicate that DNA shares epitopes with GBM constituents, but does not mediate the glomerular binding. Monoclonal anti-DNA antibodies derived from several mouse models for human SLE, such as the MRL-lpr/lpr, (NZWxNZB)F1 and graft-versus-host (GVH) mouse, have been shown to react *in vivo* with isolated glomeruli (261-266). As with the human sera, DNA could in most cases inhibit the antibody binding to isolated glomeruli, while DNaseI treatment did not affect this binding. These *in vitro* results are summarized in Table I. To approach a more *in vivo* situation of antibody binding to the glomerulus, perfusion studies have been performed in which monoclonal antibodies were directly perfused via the renal artery after which glomerular binding could be determined by immunofluorescence (Table II). Several mouse monoclonal anti-DNA antibodies, indeed, could bind to the glomerulus (264). When anti-DNA antibodies that were complexed with DNA were perfused, no binding could be observed. Also these more *in vivo* experiments revealed that human anti-DNA antibodies could bind to the GBM. After perfusion of IgG, isolated from human SLE sera by protein A-Sepharose, glomerular binding also could be observed (264). Interestingly, IgG from SLE patients with nephritis resulted in a higher intensity of glomerular binding, than IgG from SLE patients without nephritis. The binding to the GBM could be inhibited by pre-incubating the isolated IgG fraction with DNA, but not by pre-treatment with DNaseI. A different *in vivo* approach is intraperitoneal injection in im-mune-deficient mice of hybridoma cells producing a monoclonal anti-DNA antibody. This procedure leads to circulating monoclonal antibodies, which can bind to the GBM. Indeed the injection of anti-DNA hybridomas derived from MRL-lpr/ lpr (261, 265-268), (NZWxNZB)F1 (261, 264, 268) and SNF1 mice (266) resulted in glomerular deposits in the capillary wall and in the mesangium. In some cases the staining of the mesangium was more intense than the staining of the capillary walls. In another study, i.v. injection of a mouse anti-DNA monoclonal antibody also resulted in a glomerular staining pattern (265).

In conclusion, monoclonal anti-DNA antibodies, either from lupus mice or SLE patients, seem to bind to the GBM both *in vitro* and *in vivo*. However, not all antibodies are able to react with the GBM and the exact features of the antibodies, which are responsible for the GBM-associated `cross-reactivity' still remain unknown. The molecules, to which the anti-DNA antibodies bind, have been identified in some cases only.

A major component of the GBM is heparan sulfate (HS), which is the negatively charged glycosaminoglycan side chain of HS proteoglycan (HSPG). HS is responsible for the majority of the anionic sites in the GBM, which are the most important determinants for the charge-selective permeability of the GBM. Loss of

Table I. *In vitro* evidence for cross-reactivity of anti-DNA antibodies with glomerular constituents.

Species[a]	Antibody source[b]	Antibody type	Detection method	Ligand	Binding inhibited by[c]	DNaseI treatment[c]	Antibody purification	Reference
M	GVH	DNA mAb	immuno fluorescence	isolated glomeruli	ND	ND	Protein A-Sepharose	(261)
M	MRL-lpr/lpr	DNA mAb	radioactive assay	isolated glomeruli	ND	no effect	anti-Ig-Sepharose	(265)
M	MRL-lpr/lpr	DNA mAb	ELISA	laminin	DNA	ND	none	(269)
M	MRL-lpr/lpr	DNA mAb	ELISA	endothelial cell	DNA	ND	anti-Ig-Sepharose	(267)
M	MRL-lpr/lpr	DNA mAb	ELISA	HS	DNA	no effect	Protein A-Sepharose	(261, 270)
M	MRL-lpr/lpr	DNA mAb	immuno fluorescence	isolated glomeruli	ferritin	ND	Protein A-Sepharose	(261)
M	MRL-lpr/lpr	serum/ kidney eluate	ELISA	HS	DNA	no effect	none	(270)
M	(NZWxNZB)F1	DNA mAb	immuno precipitation	α-actinin	ND	no effect	Protein A-Sepharose	(268)
M	(NZWxNZB)F1	DNA mAb	immuno precipitation	isolated glomeruli	ND	ND	Protein A-Sepharose	(261)
H	SLE	serum	ELISA	HS	DNA	no effect	none	(270)
H	SLE	serum	immuno fluorescence	isolated glomeruli	DNA	no effect	none	(260)

a) Antibodies derived from mouse models (M) or SLE patients (H).
b) Mouse models are GVH, (NZWxNZB)F1 or MRL-lpr/lpr.
c) ND, no data.

Table II. *In vivo* evidence for cross-reactivity of anti-DNA antibodies with glomerular constituents.

Species[a]	Antibody source[b]	Antibody type	Method of antibody transfer	Site of deposition[c]	Proteinuria	Ligand	Antibody purification	Reference
M	GVH	DNA mAb	hybridoma inoculation	glomerular	ND[d]	HS	Protein A Sepharose	(261)
M	MRL-lpr/lpr	DNA mAb	hybridoma inoculation	capillary/mesangial	ND	ND	anti-Ig-Sepharose	(265)
M	MRL-lpr/lpr	DNA mAb	antibody injection	capillary/mesangial	ND	ND	anti-Ig-Sepharose	(265)
M	MRL-lpr/lpr	DNA mAb	hybridoma inoculation	capillary/mesangial	yes	ND	anti-Ig-Sepharose	(266)
M	MRL-lpr/lpr	DNA mAb	hybridoma inoculation	capillary/mesangial	yes	5 and 108 kDa protein	anti-Ig-Sepharose	(267)
M	MRL-lpr/lpr	DNA mAb	hybridoma inoculation	glomerular	ND	HS	Protein A Sepharose	(261)
M	MRL-lpr/lpr	DNA mAb	hybridoma inoculation	capillary/mesangial	yes	α-actinin	Protein A Sepharose	(268)
M	NZB	DNA mAb	hybridoma inoculation	none	no	ND	anti-Ig-Sepharose	(266)
M	(NZWxNZB)F1	DNA mAb	perfusion	capillary/mesangial	yes	ND	Protein A Sepharose	(264)
M	(NZWxNZB)F1	DNA mAb	hybridoma inoculation	glomerular	ND	HS	Protein A Sepharose	(261)
M	(NZWxNZB)F1	DNA mAb	hybridoma inoculation	capillary/mesangial	yes	α-actinin	Protein A Sepharose	(268)
M	SNF1	DNA mAb	hybridoma inoculation	intranuclear (glomerulus)	no	ND	anti-Ig-Sepharose	(266)
H	SLE	DNA mAb	hybridoma inoculation	capillary/mesangial	yes	ND	Protein G + high salt	(270)
H	SLE	serum IgG	perfusion	glomerular	yes	ND	Protein A Sepharose	(267)

a) Antibodies derived from mouse models (M) or SLE patients (H).
b) Mouse models are graft versus host (GVH), (NZWxNZB)F1, NZB or MRL-lpr/lpr.
c) The site of deposition is listed as glomerular if it is not specific further. If deposition occurred along the glomerular capillary wall it is listed as capillary. Mesangial represents exclusive mesangial deposition.
d) ND, no data.

anionic sites leads to proteinuria, while injection of antibodies specific for HS in rats instantly induces an acute selective albuminuria (271). In both human and murine SLE sera anti-HS reactivity has been found (270). In an early report anti-HS reactivity has been demonstrated in 30 out of 33 SLE patients that were positive for anti-DNA antibodies in the Farr assay and the anti-DNA ELISA. The observed HS binding in ELISA could not be attributed to a much higher IgG concentration in the SLE patients because the IgG concentration was only 2-fold higher compared to the control sera, while the reactivity towards HS was about 100-fold higher. To exclude any possible DNA contamination of the HS, which could easily explain the apparent binding of anti-DNA antibodies, the reactivity of human SLE sera has also been tested with HS that was pre-treated with DNaseI. However, no difference in reactivity towards HS could be observed with or without DNaseI pre-treatment (270). The titers of anti-HS-reactive antibodies showed a significant correlation with the titers of anti-DNA antibodies both in sera from SLE patients and MRL-lpr/lpr mice (270). To obtain additional evidence that the binding of anti-DNA antibodies to the GBM was mediated by HS, ELISA blocking experiments with DNA and HS have been performed (261). The binding of sera from SLE patients and MRL-lpr/lpr mice to DNA in ELISA could be inhibited by the addition of either HS or DNA. Vice versa, the HS reactivity in ELISA could also be inhibited in a dose-dependent manner by the addition of either HS or DNA. The degree of inhibition varied from 60 to 100% (261). In addition, antibodies eluted from MRL-lpr/lpr kidneys and from a kidney of a SLE patient showed reactivity towards HS, which again could be inhibited by the addition of DNA. The observed HS binding is very unlikely caused by entrapment of serum IgG in the eluate, because the reactivity was much higher in the eluates than in the corresponding serum (261). Murine monoclonal anti-DNA antibodies, that are able to bind to the GBM, also showed reactivity to HS or HSPG in ELISA. Again this could be inhibited dose dependently with DNA or HS, respectively (261). Binding of anti-DNA antibodies to isolated GBM loops also could be completely prevented by pre-incubation of the GBM loops with cationic ferritin, while pre-treatment with heparitinase (i.e. enzymatic removal of HS chains) reduced the binding considerably, but not completely. The remaining HS might be responsible for the residual binding of anti-DNA antibodies, but the result might also indicate that other anionic molecules than HS within the GBM, such as laminin, can serve as binding sites for anti-DNA antibodies (269). The glomerular binding was also not due to non-specific charge interaction, since a non-related monoclonal antibody with a high isoelectric point (above 9.0) did not bind to the GBM. The anti-DNA antibodies that resulted in glomerular deposits and showed reactivity in ELISA towards HS, were used for *in vivo* studies. Hybridomas that produced non-HS-binding anti-DNA antibodies only resulted in deposits in the mesangium. Injection of HS-binding anti-DNA producing hybridomas also led in most cases to

proteinuria in normal mice. This was not the case when non-relevant antibody-producing hybridomas were used (261). Similar experiments with hybridomas that produce human monoclonal anti-DNA antibodies did result also in a capillary wall and mesangial staining and proteinuria (272). All *in vivo* results that relate anti-DNA antibodies to glomerular deposition are summarized in Table II. All previously discussed results seem to support the theory that the most prominent autoantibody in SLE, anti-DNA, can cross-react with the negatively charged component of the GBM, HS (261). As will be outlined further this 'cross-reactivity' of anti-DNA with HS was not due to a direct binding, but mediated via nucleosomes bound to the antibody.

Screening of a random peptide phage display library is a more recent approach to identify structural motifs (2) that can explain the reactivity of anti-DNA antibodies with the GBM. This technique can be used to find mimotopes for anti-DNA (or anti-nucleosome) antibodies, which are peptide motifs that mimic the epitopes which are recognized by the antibody. The random peptide phage display technology has proven to be a powerful technique, especially for antibodies that by definition do not recognize a protein-based epitope, such as anti-DNA autoantibodies (87, 217). Briefly, the autoantibody is coated on a carrier (i.e. a plate or beads) and subsequently incubated with a phage library, which contains numerous different phages that display a short random peptide fused with a phage coat protein. Finally, the phages that bind to the anti-DNA autoantibody with the highest affinity are selected. *In silico*, the selected motifs of course can be compared to all known proteins sequences, including GBM proteins. A positive match between the sequence of the selected motif and the sequence of a GBM protein then readily explains the observed reactivity of the anti-DNA antibody with the GBM.

Several DNA mimotopes with a motif that mimics the epitope of anti-DNA autotoantibodies have been selected so far with this random peptide phage display (87-89). In one study anti-DNA antibodies from sera of SLE patients, which were positive in the Farr assay, have been purified by a γ-bind Sepharose column followed by a DNA-Sepharose column (89). The purified antibodies have been used for the screening of a phage library with a random sequence of 12 amino acids fused to the pVIII phage coat protein. After several rounds of screening a common motif (amino acid sequence: RLTSSLRYNP) could be deduced from the selected sequences. Despite the negative charge of DNA, this motif surprisingly contained some positive charges. The motif showed only homology with a chromosome-associated polypeptide. The peptide that was synthesized on the basis of the motif did react with 88% of the anti-DNA+ SLE patient sera. The reaction of the IgG used for the selection with the peptide could be inhibited by the addition of dsDNA, native RNA, denatured DNA, and most efficiently with ssDNA. Apparently, the peptide mimics a common epitope present in dsDNA, ssDNA and RNA (89).

Random peptide phage display has also been applied to several monoclonal anti-DNA antibodies. Screening of a 16-mer random peptide phage display library with three anti-DNA antibodies derived from (NZBxNZW)F1 mice did result in mimotopes for two antibodies (88). The motif obtained by one of the antibodies (F14.6) contained two cysteines, which could form a loop, negatively charged amino acids and some aromatic residues. The motif selected by the other antibody was different, but also contained two cysteines, negatively charged and aromatic amino acids. No significant homology could be found with any other proteins (88). Screening of a 10-mer pIII phage library with a mouse monoclonal anti-DNA antibody (R4A) that causes glomerular deposits in non-autoimmune mice, did also result in a mimotope (87). This mimotope contains a net negative charge, like DNA, and has two aromatic amino acids, which may mimic the sugar backbone structure or one of the base pairs. It was evaluated whether the mimotopebased synthetic peptide could inhibit the deposition of the R4A antibody to the GBM *in vivo*. Administration of only the anti-DNA antibody caused deposits in the glomerulus in SCID mice. Administration of both the mimotope-based peptide and the R4A antibody reduced the deposits in the glomerulus. Binding of the antibody to the GBM seems likely, because of the inhibition of glomerular binding by the mimotope-based peptide. Therefore, one could expect or argue that the mimotope should mimic a GBM component or structure. However, on the protein level only homology with collagen XIII could be found (C. Putterman, personal communication), a molecule that is normally not present in the GBM. The DWEYS peptide on a MAP backbone has been used to immunize 4-to 6-week-old BALB/c mice (90). The immune response resulted in antibodies against DNA, histones and cardiolipin. Most importantly, the mice developed a SLE-like disease with glomerular deposits of IgG and complement C3 (90). In a recent study the reactivity of R4A with the NR2 glutamate receptor was found (92).

Comparison of the amino acid sequences of the different DNA mimics that have been identified thus far (87-89) reveals that there is no homology between the mimotopes at all. At first, this finding may seem rather unexpected because the basic structure of naked DNA, i.e. without associated proteins, is a double helix. The differences between the several reported DNA mimics may be explained by different characteristics of the original DNA autoantigen such as different higher-order structures of DNA (supercoils) or just differences in the nucleotide sequence. Most importantly, on the amino acid level the DNA mimotopes do not show homology with any known protein in the GBM. However, it cannot be excluded that the DNA mimotopes mimic a structural feature of the GBM that cannot be easily deduced from the amino acid sequences of known proteins. In summary, several studies suggest that certain anti-nuclear antibodies can bind to glomerular components and induce glomerular deposits. However, from these studies it is difficult to conclude

which characteristics of the antibody determine glomerular binding. After a decade of research by several groups, the ligands in the glomerulus that could serve as cross-reactive epitopes for anti-nuclear antibodies still have not been identified. So the direct binding of anti-DNA antibodies to the GBM cannot yet be explained by a look-a-like of DNA present in the GBM.

After our initial observation that anti-DNA antibodies could cross-react with HS and GBM loops (270), we showed that this binding was mediated by nucleosomes bound to the anti-DNA antibodies (93). As will be outlined in the subsequent section, removal of these nucleosomal components completely abrogated the binding to HS *in vitro* and to the GBM *in vivo* (99). A similar observation has been made with respect to other cross-reactive molecules (263, 273). Given these results, in our opinion, it is likely that the majority of the observed cross-reactions, as discussed above, are mediated by nucleosomal components bound to the antibodies. Let us assume that this is the case, is it possible to explain the results described in Tables I and II. A common feature of all these experiments, summarized in Tables I and II, is that DNA can block the binding to the GBM and DNaseI treat ment has no effect. This led to the conclusion that binding of DNA to the antigen binding sites of the antibody prevents cross-reactive recognition of the GBM ligand. The lack of effect of DNaseI treatment was regarded as proof for the absence of DNA in the antigen binding site. However, these findings can be explained differently. When an anti-DNA antibody is complexed to nucleosomes, addition of DNA to these complexed antibodies will lead to binding of DNA to the positively charged histone tails, thereby neutralizing these positive charges. This masking will lead to the abrogation of binding to HS in the GBM. Addition of DNaseI to the antibodies complexed to nucleosomes will lead to the removal of DNA from the nucleosome, but will not remove the bound histone part, as we have documented (94). These remaining histone molecules are still able to bind HS in the GBM.

The nucleosomes as mediator of autoantibody binding to the GBM

The nucleosome, the fundamental unit of chromatin, is defined as the total of compacted DNA and DNA-associated biomolecules in the eukaryotic cell nucleus. In the nucleosome two superhelical turns of 146 base pairs of DNA are complexed to the pairs of four different core histones that form an octamer (Fig. 1). The core histones are histones H2A, H2B, H3 and H4, while histone H1 is bound outside of the nucleosome. The core histones have a molecular weight that ranges from about 11 to 15 kDa, a positive charge and an isoelectric point of about 11. The basic residues are clustered in the flexible N-terminal parts of the core histones,

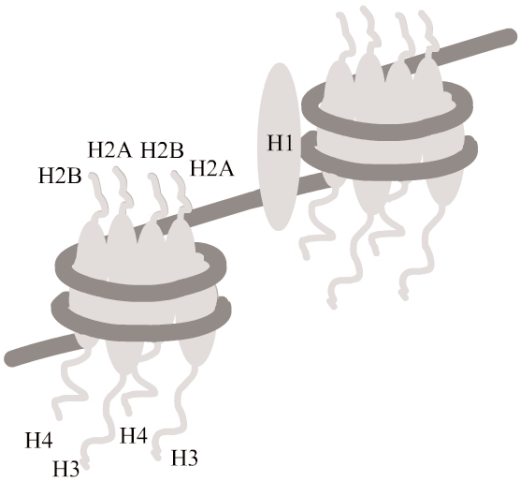


Figure 1. Composition of the nucleosome. The core particle consists of an octamer of four pairs of core histones H2A, H2B, H3 and H4, and around 146 bp of dsDNA, histone H1 is attached to the nucleosomes.

which are located outside of the histone octamer. Nucleosomes are exclusively formed during apoptosis by enzymatic cleavage of the linker dsDNA regions of chromatin. The observation that apoptosis is disturbed in SLE makes the nucleosome an important candidate autoantigen. The antigenicity of nucleosomes can probably be enhanced by modifications of the histones or DNA during apoptosis. Several types of modifications associated with apoptosis and/or chromatin condensation have been described so far. These include (1) phosphorylation or dephosphorylation of specific sites on the N-terminus of the core histones (143), (2) methylation of certain amino acids on the N-terminus of the core histones, (3) hyperacetylation or deacetylation of histones (23, 28), (4) ubiquitination so far described for topoisomerase II and histone H2A (148), (5) citrullination, the selective deamination of arginine to citrullin (149), and (6) transglutaminase-facilitated crosslinking of proteins like histone H2B (150). In addition to protein modifications, (nucleosomal) DNA can be altered by methylation.

As outlined in the introduction there is now convincing evidence that the nucleosome is the driving autoantigen in SLE. Nucleosomes are not only important in the induction phase of SLE, but they also play an important role in the development of tissue lesions. Several studies provided the evidence that nucleosomes can act as a mediator for the binding of autoantibodies to the GBM. As outlined before we found that certain anti-DNA monoclonal antibody derived from MRL lpr/lpr, (NZBxNZW)F1 and GVH mice bound to HS isolated from bovine kidney in ELISA (270). The majority of these HS+ anti-DNA antibodies also showed reactivity with HSPG isolated from human kidneys. The antibodies showed no ‘crossreactivity’ with other GBM components such as fibronectin, laminin or collagen IV. However, when the culture supernatants of the anti-DNA antibody-producing hybridomas were treated with DNaseI, the binding to HS or HSPG was strongly reduced in both ELISA and Western blot. This could mean that DNA mediates the binding of anti-DNA antibodies to HS or HSPG. The residual binding then might be explained by the insufficient removal of DNA form the antibody preparations by DNaseI

treatment, therefore further purification of the antibodies on a protein A-Sepharose column under dissociating high salt conditions was carried out. Strikingly, after this extended purification procedure all previously HS+ anti-DNA antibodies lost their ability to bind to HS as could be determined in the anti-HS ELISA, whereas they showed an unaltered anti-nuclear antibody reactivity in immunofluorescence on rat liver sections. The reactivity of these purified antibodies with HS and HSPG could be restored by the addition of the effluent of the protein A-Sepharose column. Figure 2A depicts a representative experiment with the monoclonal antibody clone #32, which later appeared to be a genuine anti-nucleosome antibody. The purified anti-DNA antibody was used for immunoprecipitation of the component that could restore the binding to HS, from the protein A effluent. After SDS-PAGE, the precipitated material clearly showed multiple bands with a molecular weight between 10 and 15 kDa, which were identical to calf thymus histones (Fig. 2B). In addition, the presence of DNA in the unpurified antibody preparation could be detected by agarose gel electrophoresis after DNA isolation (Fig. 2C). DNaseI treatment of the protein A-Sepharose column effluent abolished the reconstitutive effect of the effluent with respect to HS binding of purified anti-DNA antibodies, which provided additional evidence for the presence of bound DNA in unpurified anti-DNA antibodies. The presence of both DNA and histones was required for the binding of anti-DNA antibodies to HS because the separate addition of histones or DNA to the purified antibody could not restore the HS binding. Apparently, a DNA-histone complex was already bound to the unpurified anti-DNA antibody. Indeed, during hybridoma culture nucleosomes are released (274). We therefore concluded that the binding of anti-DNA antibodies to HS and HSPG is mediated by DNA-histone complexes bound to the anti-DNA antibody (93).

In addition to the ELISA studies, *in vitro* binding studies have been performed with isolated GBM loops (275). Anti-DNA antibodies extensively purified as described above did not bind *in vitro* to the GBM loops isolated from human kidney. Pre-incubation of the anti-DNA antibodies with both DNA and histones led to a strong granular binding. Addition of histones or DNA alone to the anti-DNA antibodies did not lead to binding to GBM loops. Pre-incubation of GBM loops with cationic ferritin prevented subsequent binding of histone-DNA-antibody complexes, which indicated that anionic sites like HS in the GBM were important for this binding.

To analyze whether this binding to HS could also take place *in vivo*, renal perfusion studies in normal rats have been performed. In this approach histones, DNA and purified antibody were subsequently perfused via the renal artery (Fig. 2, D and E). Perfusion of non-complexed purified antibodies did not lead to glomerular binding, however, after successive perfusion of histones, DNA and several anti-

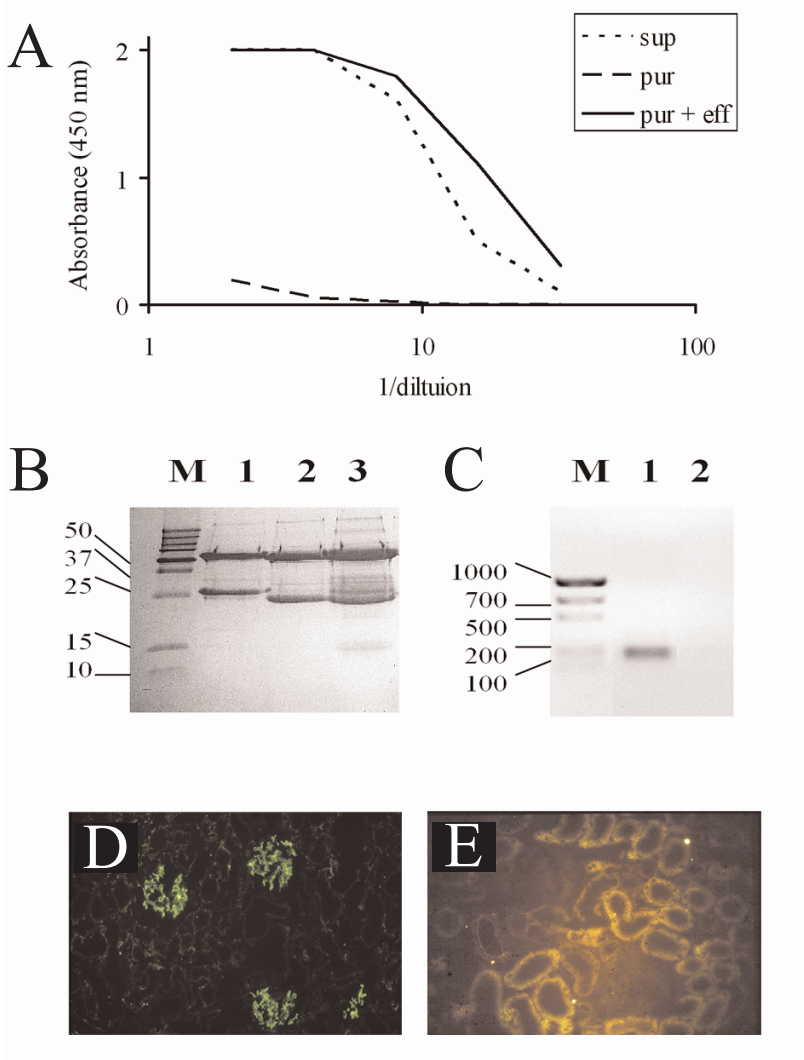


Figure 2. Reactivity with HS and the GBM of anti-nucleosome antibody #32. The purification procedure included DNaseI treatment and Protein A-Sepharose chromatography under dissociative high salt conditions. **A.** Clone #32 binds in the HSPG ELISA before purification (sup); after purification under dissociative condition on a Protein A column this reactivity is lost (pur), while addition of the Protein A column effluent to the purified monoclonal antibody restores the binding activity (pur + eff). **B.** SDS-PAGE followed by silver staining of antibody fractions before purification (lane 3) and after purification (lane 1 and 2). Before purification histone bands are visible. Marker (M) is a protein marker (in kDa). **C.** Agarose gel electrophoresis after DNA extraction of antibody fractions before (lane 1) and after purification (lane 2). Marker (M) is a DNA marker (in bp). The gel is stained with ethidium bromide. **D.** Immunofluorescence (stained with FITC-labeled anti-IgG) of rat kidney sections. After successive renal perfusion of histones, DNA and purified monoclonal antibody #32 into the left kidney. A strong staining of glomeruli can be detected. **E.** Similar to (D), but now histones, DNA and a non-relevant antibody (WT32) have been successively perfused. No glomerular staining is seen (glomeruli indicated with arrow). Note that due to prolonged exposure auto-fluorescence of the tubuli is observed.

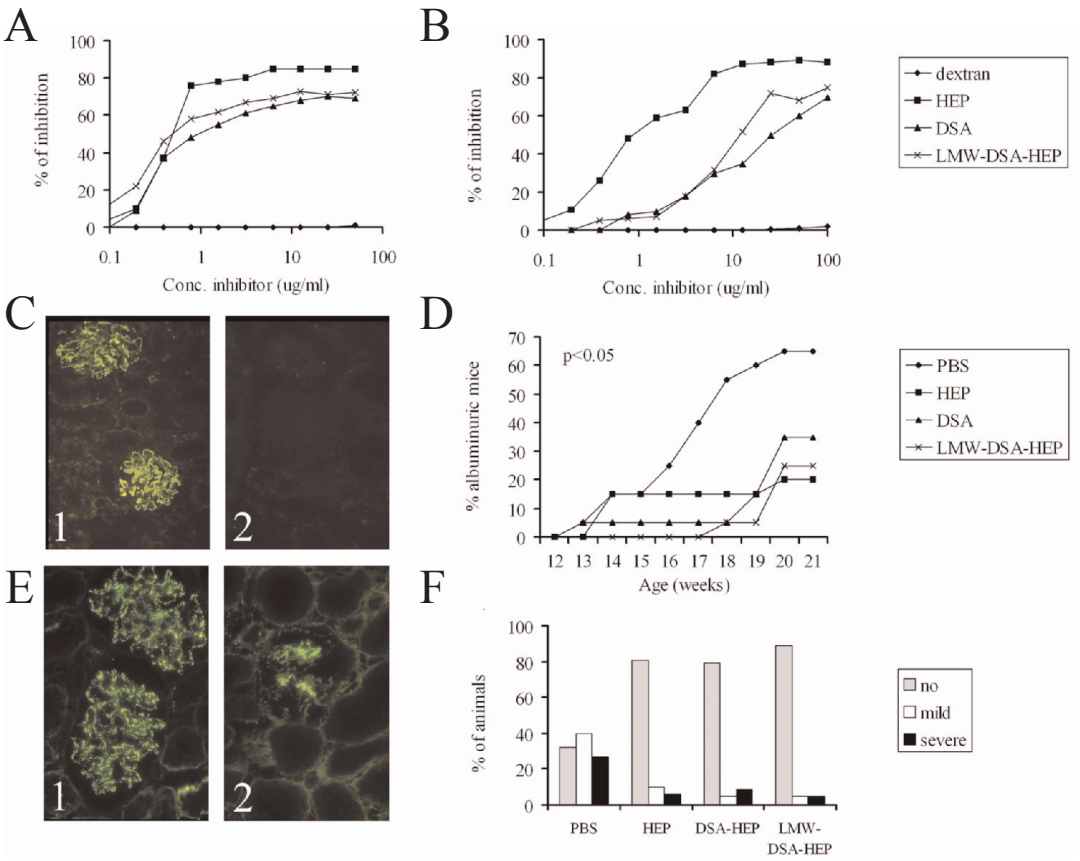
DNA antibodies, immunofluorescence analysis revealed an intense staining of the glomeruli, arteries and peritubular capillaries. Immunoelectron microscopy analysis showed large deposits at the cell membranes of glomerular endothelial cells and smaller complexes in the GBM. In the case of a high-avidity anti-DNA monoclonal antibody, perfusion of only DNA and subsequently an anti-DNA antibody did only result in mesangial deposits, but not in deposits in the capillary loop.

A completely different glomerular deposition pattern could be observed when the high avidity anti-DNA antibody was administered into the tail vein 1 h after the perfusion of histones and DNA into the left kidney via the renal artery and subsequent restoration of the renal circulation (275) Deposits now could be detected along the capillary wall in a membranous pattern, while variable amounts were present in the mesangium. Immunoelectron microscopy revealed the presence of immune deposits in the GBM located in the slit pores and under the foot processes of the podocytes. In contrast to the direct perfusion method, no binding to the endothelial cells could be observed. Most likely the 1 h time interval between intrarenal perfusion of histones-DNA and the intravenous administration of the anti-DNA antibody allows for the penetration of DNA-histones into the glomerular capillary wall, where they then serve as planted antigens for the monoclonal anti-DNA antibody. The direct perfusion method also resulted in an increased urinary excretion of albumin, which suggests a damaged glomerular filter. Control experiments with non-related monoclonal antibodies of the same isotype and at the same concentration did not result in any glomerular binding. In conclusion, anti-DNA antibodies can bind via complexes of histones and DNA to the GBM, which results in subendothelial or subepithelial deposits and proteinuria. Only in the case of high-avidity anti-DNA antibodies was the presence of DNA alone sufficient for glomerular binding, which, however, was exclusively localized in the mesangium. These studies revealed that the DNA-histone complex served as a planted antigen in the GBM for anti-DNA antibodies (275). Histones alone can also act as planted antigens for artificially prepared DNA-anti-DNA complexes as has been shown in a study in rats, in which prior to the administration of DNA-anti-DNA complexes, histones were perfused through the renal artery (276).

In vivo histone-DNA complexes exist as nucleosomes, therefore, the perfusion experiments described above were repeated with antibodies complexed with nucleosomes (94). The applied monoclonal antibodies were partially purified from the hybridoma culture supernatant by protein A-Sepharose under physiological salt conditions, which yields antibodies still complexed to nucleosomes. Control perfusions were performed with purified antibodies free of nucleosomes (see also Fig. 2, B and C). This latter purification method included DNaseI treatment of the hybridoma culture supernatant, a protein A-Sepharose column under high salt

Figure 3. Effect of heparin and non-coagulant heparinoids on binding of nucleosome/anti-nucleosome complexes to HS or the GBM. *In vitro* experiments: inhibition of the binding of the complexed anti-nucleosome antibody #34 to coated HS (**A**) or DNA (**B**) in the inhibition ELISA. Binding of complexed #34 to both HS (**A**) and DNA (**B**) was inhibited dose dependently by heparin (HEP), N-desulfated/acety-lated heparin (DSA-HEP) and low-molecular-weight desulfated/acetylated heparin (LMW-DSA-HEP). Dextran could not inhibit the binding. *In vivo* experiments: **C.** Direct immunofluorescence (with FITC-labeled anti-IgG) of glomeruli of BALB/c mice after perfusion of complexed anti-nucleosome #34 mixed with dextran (1) or heparin (2). After perfusion of complexed antibody #34 mixed with dextran clear binding of the complex to the glomerulus was observed (1). However, the addition of heparin to the complexed antibody #34 could completely prevent this binding (2). **D.** Cumulative incidence of albuminuria in the various groups of MRL-lpr/lpr mice. Starting at the age of eight weeks MRL-lpr/lpr mice were treated once daily with either 50 µg HEP, DSA-HEP, LMW-DSA-HEP or with PBS as control. Each group consisted of 15 animals. Albuminuria was considered to be present when the urinary albumin excretion exceeded 300 µg/18 h (mean +2 SD of albuminuria in non-SLE normal control mice). This mean value is 100 µg/18 h, $P < 0.05$. **E.** Representative examples of the immunofluorescence findings of glomeruli of MRL-lpr/lpr mice either treated with PBS (1) or HEP (2). PBS-treated mice show extensive deposition of IgG, mainly along the capillary wall. In HEP-treated animals the deposits were confined to the mesangium. Thus, HEP treatment prevented deposition in the capillary loop. **F.** Severity of glomerular lesions in MRL-lpr/lpr mice treated with phosphate-buffered saline (PBS), HEP, DS-HEP or LMWHEP. The severity of the glomerulus was scored as normal (no), mild glomerulonephritis or severe glomerulonephritis.

conditions and in some cases a DNA-cellulose column, which removes antibodies still bound to histones. The purity of the antibody fractions was analyzed by SDS-PAGE and agarose gel electrophoresis for the presence of histones and DNA, respectively. In contrast to unpurified antibodies and antibodies purified under physiological conditions, all extensively purified antibodies did not contain DNA and/or histones. The purified antibodies have been tested in ELISA for their reactivity towards DNA, histones and nucleosomes. It then turned out that only some anti-DNA antibodies reacted with nucleosomes. Most likely, the reactivity with dsDNA of the formerly classified anti-DNA antibodies was due to histones within the nucleosome bound to the antibody. The `genuine' anti-DNA antibodies reacted after purification with both DNA and nucleosomes. The reactivity of `anti-DNA antibodies', which were in fact anti-nucleosome antibodies, with HS in ELISA could only be demonstrated when these antibodies were not purified. In agreement with this latter finding, the purified anti-nucleosome antibodies did not show glomerular binding, while unpurified anti-nucleosome antibodies did show glomerular binding, as could be seen by immunofluorescence after renal perfusion experiments. As expected, reconstitution of purified anti-nucleosome antibodies with purified calf thymus nucleosomes restored glomerular binding. In conclusion, both immune complexes consisting of anti-DNA-nucleosome and anti-nucleosome-nucleosome are able to bind to the glomerulus *in vitro* and *in vivo* (94). To



investigate the significance of HS in the GBM for glomerular binding of nucleosome-complexed autoantibodies, heparinase was perfused intrarenally prior to administration of the immune complexes. After heparinase perfusion the staining of HS side chains of HSPG within the GBM almost disappeared completely, while the staining of the HSPG core protein was not affected. Staining of other GBM components, like laminin and collagen IV also was not affected by heparinase perfusion. Perfusion of nucleosome-autoantibody complexes after the heparinase treatment did result in a decreased binding to the GBM, but this binding did not disappear totally (94). This observation suggested that HS is not the only ligand that is responsible for the binding of anti-nucleosomes-nucleosome immune complexes to the GBM. The binding of nucleosome-containing immune complexes to other GBM components has indeed been reported (277). In this latter study, the binding of sera from MRL-lpr/lpr mice to GBM could be competitively inhibited by DNA or histones and collagenase treatment of the GBM. The binding of the antibodies could only be restored after the addition of collagen IV. With purified GBM components coated in ELISA antibody binding could only be observed when DNA was coated on collagen I; however, no or little binding was observed when DNA was coated on

collagen IV, laminin or fibronectin. Coating of histones prior to DNA coating on collagen IV resulted in a positive binding of the MRL/lpr sera. These latter findings suggested that nucleosomes can bind to collagen IV via their histone parts, analogous to the situation with HS (277).

Other evidence that HS is the major target in the GBM for nucleosome-mediated autoantibody binding came from our studies in which we used heparin, which is structural very similar to HS (174). We hypothesized that heparin could bind to the positively charged N-terminal parts of the core histones within the nucleosomes, thereby preventing histone binding to HS. Indeed, in ELISA heparin and non-coagulant heparinoids could inhibit dose-dependently the binding of nucleosome-complexed autoantibodies to HS or DNA (Fig. 3, A and B). Similarly, in renal perfusion experiments heparin could prevent glomerular binding of nucleosome-complexed autoantibodies, while dextran showed no effect (Fig. 3C). *In vivo* in MRL-lpr/lpr mice daily subcutaneous administration of heparin or non-coagulant heparin derivatives prevented the development of proteinuria and glomerular lesions (Fig. 3, D and F). Immunohistology of the kidneys revealed that in heparin(oid)-treated mice only mesangial deposits were present and in 80% of the heparin(oid)-treated animals no glomerular lesions could be observed (Fig. 3E) (174). These results support that immune complex deposition in lupus mice is mediated for the greatest part by the interaction of cationic histones with anionic HS. To further document that the N-termini of the core histones are important for the binding to HS, we performed *in vivo* inoculation studies with hybridoma cells in BALB/c nude mice (82). We hypothesized that anti-histone producing hybridomas would generate less nephritogenic nucleosome-autoantibody complexes than anti-nucleosome or anti-DNA antibodies. Our hypothesis was based on the fact that the epitopes for anti-histone antibodies reside within these N-termini. Binding of anti-histone antibodies to the cationic tails would reduce the ability of histones to bind to the anionic HS in the GBM, i.e. because of masking of the positive charges on these N-termini. Binding of anti-nucleosome or anti-DNA antibodies would hardly influence these charges. Inoculation of the different hybridoma cells led to detectable levels of antibodies in ascites only against the part of the nucleosome, to which the antibody produced by the hybridoma was directed (Fig. 4A). Antibody reactivities in plasma samples were comparable to those obtained in ascites indicating that the antibodies were transferred to the systemic circulation. Indeed, inoculation of mice with three different anti-histone hybridomas induced only in 15% of the animals glomerular deposits, while with three different anti-nucleosome and three anti-DNA antibodies 60% of the animals had GBM deposits (Fig. 4, C and D) (82). Apparently, the differences in glomerular binding between the antibodies were not due to differences in the levels of nucleosome-autoantibody complexes,

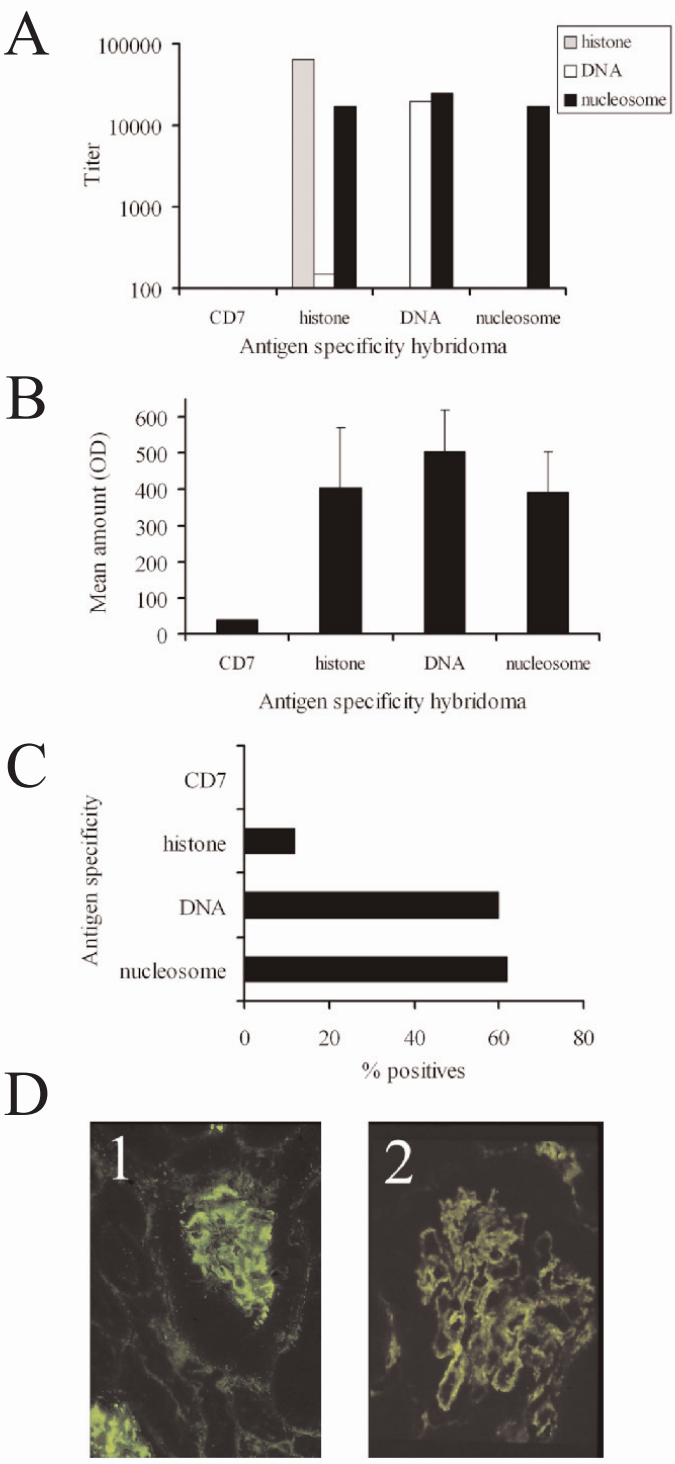


Figure 4. Glomerular binding after intraperitoneal inoculation of hybridomas producing anti-nuclear antibodies. **A.** Reactivity of ascites of mice inoculated with anti-DNA, anti- histone, anti-nucleosome or control hybridoma (producing CD7) in ELISA towards the different nuclear antigens: histones, DNA and nucleosomes. The titer in ELISA was defined as the reciprocal of the dilution giving an OD of 1.0 at 450 nm and expressed per mg of Ig. The results show that the appropriate specificity was detected in the ascites.
B. Nucleosome-IgG complex assay performed on as cites from mice inoculated with anti-DNA (n =3), anti-histone (n =3), anti-nucleosome toneal (n =3) or CD7 control hybridomas (n =3). Values are given as mean ± SEM. No statistically significant difference was found between the amounts of complexes formed for the different anti-nuclear antibodies. **C.** Immuno-fluorescence analysis of kidney sections of mice inoculated with anti-DNA (n =15), anti-histone (n =13), anti-nucleosome (n =13) or control hybridomas (n =6). The results are expressed as percentage of mice with GBM deposits. **D.** Representative examples of kidney sections of mice inoculated with anti-DNA #42 (1) or anti-nucleosome #32 (2).

since these were not different (Fig. 4B).

These latter observations may seem in contrast with previous results that show direct binding of anti-DNA antibodies to the GBM, which were explained by cross-reactivity. However, there is a good explanation for this apparent contradiction. A major point of concern is the purity of the antibody fractions that have been used in the experiments as outlined earlier. During the culture of hybridoma cells apoptosis occurs spontaneously and of the hybridoma cells nucleosomes are released (274). This material will subsequently bind to the monoclonal antibodies that are present in the supernatant and which are directed against nucleosomes. This undoubtedly leads to the formation of nucleosome-antibody complexes (94, 278). In studies describing cross-reactivity mostly unpurified antibodies from culture supernatants or only partial purified antibodies by protein A/G-Sepharose columns are used. However, this latter purification step under physiological conditions does not dissociate the bound DNA and especially the bound histones from the antibody, as we have shown (94). Sometimes a DNaseI treatment of the culture supernatant is used, but in most cases not in combination with high salt-p rotein A/G-Sepharose. In our experience anti-nucleosome and anti-DNA antibodies have to be purified by DNaseI treatment, high salt-protein A/G-Sepharose and a DNA cellulose column (which removes residual histone-containing complexes). Many reports do not show or discuss the purity of the applied anti-DNA, anti-histone or anti-nucleosome antibody preparations, which is of prime importance for studies that aim to unravel the mechanism of glomerular deposition in SLE.

Evidence for nucleosome-mediated binding in lupus nephritis

So far, we have presented experimental data for the role of nucleosomes as mediators for autoantibody binding to the GBM. However, if this concept is true, nucleosomes and anti-nucleosome antibodies should be present in the glomeruli of patients and mice with spontaneous lupus nephritis. We therefore set up a number of studies to investigate whether these proteins and antibodies were present in the glomerulus in lupus nephritis. First, we searched for the presence of nucleosomes/nucleosomal antigens in glomerular deposits. A polyclonal anti-histone H3 serum which reacts with the N-terminal amino acids 1-21 was used to probe histones in kidney biopsies of 11 SLE patients with diffuse proliferative glomerulonephritis (DPGN) and six patients with lupus membranous glomerulonephritis (MGN) (95). Histones could be detected in all of the eleven SLE patients with DPGN and two out of the six SLE patients with MGN (Fig. 5A). A histone monoclonal antibody (KM-2), which reacts with the N-terminal parts of both histone H2A and H4, only demonstrated the presence of histones in three patients

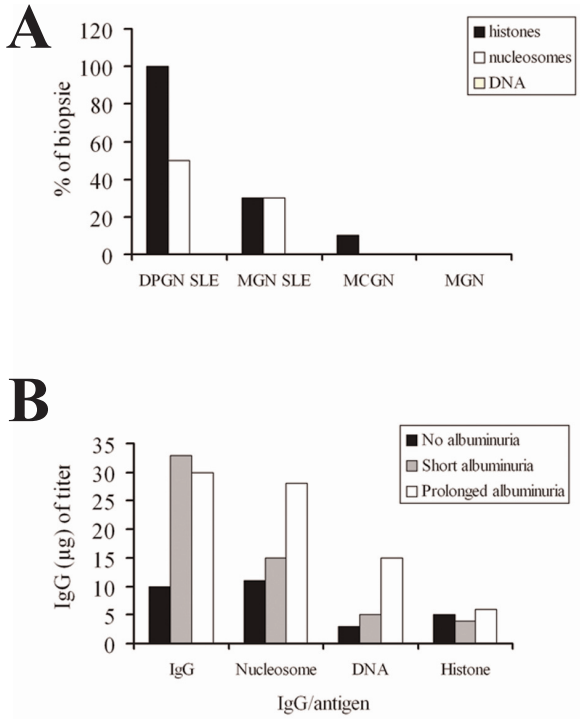


Figure 5. Presence of nucleosomes and anti-nuclear antibodies in glomerular deposits in lupus nephritis. **A.** Presence of nucleosomal antigens in GBM deposits in kidney biopsies of SLE patients with either DPGN or MGN and non-SLE controls with either mesangiocapillary glomerulonephritis (MCGN) or idiopathic MGN. Staining of histones with polyclonal rabbit anti-H3 1-21 serum, staining of nucleosomes with monoclonal antibody #34, LG8-1 and LG10-1, staining for DNA with antibody #42 was negative in all biopsies.
B. Immunoglobulin concentration and titers towards nucleosomal antigens of glomerular eluates from MRL-lpr/lpr mice. The 18-to 24-week-old mice either had no albuminuria, short albuminuria of less than 7 days or prolonged albuminuria for 14-21 days.

with DPGN and none of the patients with MGN. In addition a nucleosomal staining could be detected by using three different nucleosome-specific antibodies (LG10-1, LG8-1 and #34) in five patients with DPGNand two patients with MGN(the same two patients that stained positively for histones). Anti-DNA antibodies (#36, #42 and #56) did not reveal DNA in glomerular deposits. This latter finding seems remarkable since nucleosomes (that contain DNA per definition) could be detected by anti-nucleosome antibodies. We assume that all epitopes within DNA recognized by the probing anti-DNA antibodies were masked by autoantibodies present within the glomerular deposits. This also explains why the histone epitopes were much more easily to detect, because when these epitopes are covered by anti-histone antibodies the complex will not bind to the GBM as outlined above. In non-SLE glomerulonephritis none of the applied monoclonal antibodies could stain glomerular deposits. However, the presence of DNA in immune deposits associated with human lupus nephritis has been demonstrated at the electron microscopy level by an anti-DNA antibody labeled with protein A-gold and DNaseI-gold complexes (279). Histones and nucleosomes have also been detected in immune deposits in two models of murine SLE (NZBxNZW)F1 and GVH mice (280).

In order to evaluate the sequence of deposition of the different autoantibody specificities in MRL-lpr/lpr mice, we eluted glomeruli from 18-to 24-week-old mice with either no proteinuria, short duration proteinuria (within 1 week of onset) or heavy proteinuria (for more than 3 weeks) (248). The onset of proteinuria was

accompanied by a 3-fold higher IgG content of the eluate, which did not increase further in mice with heavy proteinuria. The analysis of the antigen specificities in the eluates showed that anti-nucleosome antibodies deposited first, while the highest reactivity for anti-DNA antibodies was found in mice with heavy proteinuria. Anti-histone reactivity was low and did not increase further when proteinuria developed or progressed (Fig. 5B).

Evidence for the presence of nucleosome-immune complexes in lupus nephritis was obtained rather indirectly. Using monoclonal antibodies against HS and HSPG core protein (271, 281) we found an almost complete absence of HS staining in the GBM in renal biopsies from patients with lupus nephritis, while the staining for the core protein remained unaltered (282). Since MRL/lpr mice also showed this decrease in HS staining at advanced states of nephritis (283), we could study the responsible mechanisms in more detail. In these lupus mice the decrease in HS staining correlated inversely with the amount of immunoglobulin deposits in the GBM. The pathophysiological significance of the reduction of HS staining was underlined by the inverse correlation with albuminuria. Also in human lupus nephritis an inverse correlation was found between HS staining and the amount of

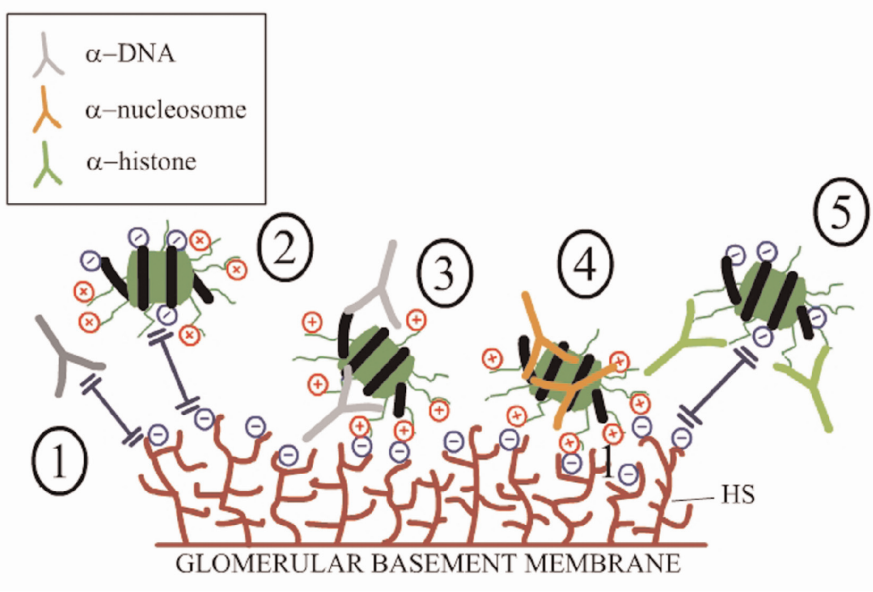


Figure 6. Schematic representation of the binding of nucleosome-anti-nucleosome complexes to the GBM. (Left to right) No binding will occur of non-complexed anti-nuclear antibodies (**1**) or free nucleosomes (**2**). Binding of anti-nucleosome (**3**) or anti-DNA (**4**) will decrease the density of negative charges of the nucleosome - this will enhance binding of the complex to the negatively charged GBM. In contrast to this, binding of anti-histone antibodies (**5**) to the nucleosome will decrease the amount of positive charges, which reduces the capacity to bind to HS in the GBM.

histone deposits in the GBM (95). The decrease in HS staining was not due to decrease in HS content since the amount of HS within the GBM was normal (283). Together, these data suggest that the decrease in HS staining is due to masking of HS by nucleosome-immune complexes. Indeed, nucleosome autoantibody complexes were able to inhibit dose dependently the binding of the anti-HS monoclonal antibody JM403 to HS in ELISA, whereas non-complexed autoantibodies had no effect (283). As outlined above, heparin treatment in MRL/lpr mice prevented formation of deposits in the GBM. In these heparin-treated animals a decrease in HS staining was not observed (174). Therefore, in SLE nephritis HS is masked by deposited nucleosome-autoantibody complexes. This masking may have the same functional consequences as reduction of HS, since it may lead to an enhanced permeability of the GBM as shown by the induction of albuminuria by the anti-HS monoclonal (271).

Conclusion

Nucleosome-mediated autoantibody binding to HS in the GBM is responsible for the formation of immune deposits in the GBM in lupus nephritis. As outlined in this review, a large part of the evidence for direct binding of autoantibodies to the GBM due to `cross-reactivity' is questionable and is explained by the use of unpurified autoantibodies, more specifically the use of autoantibodies already complexed with nucleosomes. In our model, detailed in Fig. 6, autoantibody binding or deposition to the anionic GBM is mediated by the binding of the cationic and protruding N-termini of the histones to the GBM. Furthermore, the binding of anti-nucleosome or anti-DNA antibodies to the nucleosome will mask its negative charge and will facilitate the binding of the positively charged N-terminal histone tails to the GBM, which ultimately leads to the deposition of immune complexes. On the other hand the binding of anti-histone antibodies to the N-terminal histone parts will mask their positive charge, which will lead to reduction of deposition of immune complexes (see Fig. 6 for details).

Chapter 10.

Summary and future directions of research

The autoimmune disease systemic lupus erythematosus (SLE) is characterized by autoantibodies that target about 100 sofar identified targets (8). The nucleosome has been suggested as a major player in this complex autoimmune response (39). Nucleosomes are the basic structure of chromatin, which enables the dense packing of DNA in the nucleus. Each nucleosome consists of 146 base pairs of dsDNA wrapped twice around an octamer of pairs of the core histones H2A, H2B, H3 and H4. Chromatin is cleaved into nucleosomes during apoptosis and clusters together with other autoantigens at the cell surface of apoptotic cells. During apoptosis, nucleosomes can be modified by the addition or removal of covalently linked moieties. Therefore, we appointed a central role to the nucleosome which links an impaired apoptosis or removal of apoptotic cells and the subsequent release of modified apoptotic material to the initiation of an anti-chromatin autoimmune response and the development of lupus nephritis (see also Chapters 1 and 2). In this thesis, we have further evaluated the role of the nucleosome within this proposed model.

Apoptosis-induced histone modifications: the driving force for the anti-chromatin response in SLE

Apoptotic cells are the unique source of nucleosomes, which are formed through the cleavage of chromatin by endonucleases. Apoptosis, or programmed cell death, involves cleavage and modification of numerous proteins. Remarkebly, many of these modified non-histone proteins are targeted by SLE autoantibodies. Although, apoptosis-induced histone modifications have been described, these modifications have never been identified as targets for SLE-derived autoantibodies. In **chapter 3**, we have identified apoptosis-induced acetylation of histones as a target for SLE autoantibodies. We mapped the epitope of KM-2, a monoclonal anti-histone autoantibody derived from the MRL/lpr lupus mouse, by random peptide phage display in the N-terminal region of histone H4. For this region acetylation of lysines at positions 5, 8, 12 and 16 has been described. We found that KM-2 preferentially recognized H4 peptides acetylated at lysine 8,12 and/or 16. Moreover, we assessed the binding of KM-2 to histones from cells treated with a histone deacetylase inhibitor (Trichostatin A, TSA), which induces hyperacetylation of histones, and cells treated with camptothecin (CPT, an inhibitor of DNA topoisomerase I) or anti-Fas antibody, which both induce apoptosis. Cells treated with TSA, CPT or anti-Fas all revealed a stronger nuclear staining with KM-2. This was also confirmed by Western blot analysis of the resolved extracts, which revealed

an enhanced reactivity of KM-2 with histone H4 and H2A. In addition to these findings with the monoclonal autoantibody KM-2, we also found that polyclonal autoantibodies in plasmas from SLE mice and patients showed an increased reactivity with at least one of the acetylated H4 peptides compared to the unmodified H4 peptide. In addition, Western blot analysis revealed a higher reactivity of these plasmas with histones H4, H2A, H2B, H3 (and H1) extracted from cells treated with CPT or TSA. Acetylation and deacetylation of histones is a dynamic process, which involves histone acetyltransferases (HATs) and histone deacetylases (HDACs). The apoptosis-induced hyperacetylation of histones appeared to be mediated by an increased histone acetyltransferase activity and a decreased histone deacetylase activity as measured by a fluorescence-based assay in (apoptotic) cell extracts. From these results we concluded that histones acetylated during apoptosis are a target of autoantibodies in SLE.

To asses the effect of a tri-acetylated histone H4 peptide on the development of disease in MRL/lpr mice, we treated these mice with either a histone H4 peptide tri-acetylated at lysine residue 8, 12 and 16, an unmodified H4 peptide or PBS. Treatment of MRL/lpr mice with the tri-acetylated H4 peptide, compared to treatment with unmodified H4 peptide or PBS, significantly accelerated disease as measured by proteinuria and the progression of skin lesions. The titers of the anti-chromatin autoantibodies were not different between the different treatment groups, but the delayed type hypersensitivity (DTH) response against the tri-acetylated H4 peptide was significantly higher than against the unmodified H4 peptide in all groups, irrespective of the treatment. Therefore, in the MRL/lpr lupus mice a T cell response is mounted against these histone modifications.

These novel observations indicate a crucial role of apoptosis-induced histone modifications in the pathogenesis of SLE. Apoptosis-induced autoantigen modifications are normally not encountered by the immune system. However, an impaired removal of apoptotic cells leads to the release of modified nucleosomes (see next section). This may then lead to the disruption of the balance between tolerance and autoimmunity.

Removal of apoptotic cells: release of modified nucleosomes

Normally, phagocytes (mainly macrophages) swiftly remove apoptotic cells to prevent exposure of their contents to the environment. Accumulating evidence shows that in SLE a defective removal of apoptotic cells leads to the release of apoptotic material, including modified nucleosomes and histones. Mice deficient for molecules involved in the complex process of apoptotic cell removal, develop anti-nuclear autoantibodies and in some cases glomerulonephritis. In SLE patients, it

been demonstrated phagocytosis of apoptotic cells is impaired. However, in lupus mice, like the MRL/lpr and (NZWxNZB)F1 models, there is no clear evidence for an impaired phagocytosis of apoptotic cells. Previously, work from our lab has demonstrated that a defective removal of apoptotic cells is not an intrensic defect already present in lupus mice before disease development (42). In **chapter 4**, we describe a decreased removal of apoptotic cells in diseased SLE mice. We analysed the ability of resident peritoneal macrophages from pre-diseased and diseased MRL/lpr and (NZWxNZB)F1 mice to phagocytose apoptotic cells in an *in vitro* assay. The *in vitro* assay was carried out with autologous macrophages and apoptotic thymocytes in the presence of autologous serum. Macrophages isolated from diseased MRL/lpr and NZBxNZW(F1) SLE mice, and from age-matched NZB mice showed a decreased phagocytic capacity. The decreased phagocytosis was due to a factor present or absent in the serum of diseased SLE mice, since the addition of serum from diseased mice to macrophages from normal or pre-diseased lupus mice led to a decreased of phagocytosis. In contrast to another report (43), we could not demonstrate a constitutive phagocytic defect in the macrophages of pre-diseased or diseased SLE mice. However, we did find a difference in phagocytic capacity for apoptotic cells when we compared macrophages from several control mice strains. This may explain the results described in the studie of Potter and co-workers, which used macrophages BALB/c mice as a control, which appeared to possess a higher phagocytic capacity compared to other control and SLE strains.

We concluded that during disease progression in murine lupus apoptotic cell clearance deteriorates due to a factor present or absent in the serum, which may play an inportant role in disease progression. This serum factor may be a critical component required for the phagocytosis or an inhibitor of phagocytosis. However, further research is needed to identify this serum factor. Several candidates involved in the removal of apoptotic cells are of potential interest (40), such as IgM, complement, DNaseI, serum amyloid P (SAP), thrombospondin, C-reactive protein, C1q, nucleosomes (39). An alternative explanation could be the presence of certain autoantibodies which influence this process.

Anti-nucleosome & anti-C1q autoantibodies in patients with lupus nephritis

Anti-nucleosome autoantibodies are most frequently found in SLE patients. The level of anti-nucleosome autoantibodies appears to be correlated with disease activity and the presence of lupus nephritis, the most severe manifistation in SLE patients (41). Moreover, anti-nuclear autoantibodies can be detected in the sera of patients already up to 10 years before onset of the disease (164). Anti-nucleosome

autoantibodies can be divided into three categories: 1) antibodies that recognize DNA (anti-DNA); 2) antibodies that recognize histones (anti-histone); and 3) antibodies that exclusively or predominantly recognize nucleosomes or subnucleosomal structures (nucleosome-specific). Within the Dutch Working Party on SLE, we have studied the anti-nucleosome, anti-DNA and anti-histone response in SLE patients with proliferative lupus nephritis (**chapter 5**). These patients were enrolled in the first Dutch lupus nephritis trial and were either treated with cyclophosphamide and prednison (CY) or azathioprine, methylprednisolon and prednison (AZA). This latter treatment was compared to the standard CY/P treatment, since cyclophosphamide often results in infertility in these young female lupus patients. We analysed autoantibody profiles in these patients during their first year of treatment. We could demonstrate that there was no significant difference in the anti-nucleosome, anti-DNA, anti-histone or anti-C1q responses between the two treatment groups. We observed anti-nucleosome, anti-DNA, anti-histone anti-C1q reactivity in 81%, 96% , 23% and 62% of the patients, respectively. Antibody titres rapidly decreased for all autoantibodies shortly after start of the treatment. We found a correlation between anti-nucleosome, anti-DNA and anti-histone reactivity, but not with anti-C1q reactivity. Anti-nucleosome and anti-DNA antibodies correlated with disease activity. We conclude that almost all patients with proliferative lupus nephritis are positive for anti-nucleosome and/or anti-DNA autoantibodies, with no clinical differences between patients with high or low antibody titers. Treatment with cyclophosphamide or azathioprine induced a comparable decline in anti-nucleosome, anti-DNA and anti-C1q autoantibodies.

Mimotopes for nucleosome-specific autoantibodies: nucleosomes interfere in *in vitro* assays

Although nucleosome-specific autoantibodies already are present early in disease, even before anti-DNA autoantibodies arise, and are involved in the initiation if lupus nephritis, their specific detection is very complex, since the use of nucleosomes as coating results also in the detection of anti-DNA and anti-histone autoantibodies. Therefore, mimopes for nucleosome-specific autoantibodies, i.e. peptides micking conformational nucleosomal epitopes, could be very usefull for this purpose. Analogous to mimotopes for anti-DNA autoantibodies identified previously, we have selected and identified a mimotope for a monoclonal lupus-derived nucleosome-specific autoantibody (mAb #32) by screening a random peptide phage display library (**chapter 6**). The sequence NRKDWIERTL (called MIMO#0) contained both negatively (D,E) and positively (R,K) charged residues, possibly mimicking DNA and histones, respectively. To our surprise and regret the

identified mimotope turned out to be a DNA-binding motif instead of the epitope for mAb #32. Firstly, in ELISA we discovered that reactivity of mAb #32 to the MIMO#0 peptide was dependent on the blocking reagent. Secondly, we encountered that in addition to mAb #32, most monoclonal nucleosome-specific, anti-DNA and anti-histone autoantibodies recognized the MIMO#0 peptide, which again was dependent on the applied blocking reagent. Thirdly, DNaseI treatment of the blocking reagent totally abolished the reactivity of all monoclonal antibodies. Fourthly, we could directly demonstrate that DNA and histones were present in several blocking reagents. Finally, the MIMO#0 peptide could directly bind nucleosomal material (i.e. DNA /histones) and labelled DNA. Importantly, we obtained similar results for a DNA mimotope previously identified by Sun and co-workers (89). So nucleosomal material, present in experimental reagents, such as blocking reagents and bacterial growth media, that are used during the selection and characterization of the mimotopes, prevent the identification and proper characterization of mimotopes for anti-DNA and nucleosome-specific antibodies. Besides the reactivity of monoclonal antibodies, we found that nucleosomal material in blocking reagents not only affected the reactivity of patients sera with mimotopes, but also with DNA, histones and nucleosomes. So far, based on our results specific blocking reagents without nucleosomal material should be used when these anti-nuclear autoantibodies are measured. Although such a blocking reagent does not yet exist, we found that for analysis of anti-DNA and anti-nucleosome autoantibodies, FCS is an acceptable blocking reagent, while for anti-histone Dnase-treated milk can be used. However, the use of these blocking reagents in these assays will not completely solve the problem. Therefore, the search for blocking reagents without DNA and histones should be continued. Moreover, it is questionable whether with the random peptide phage display technique mimotopes for anti-chromatin autoantibodies can be identified, despite the fact that with this technique mimotopes were identified for non-nucleosomal non-protein structures (215).

An anti-histone autoantibody specific for histone variant H3.1

In contrast to the identification of mimotopes for nucleosome-specific autoantibodies described in chapter 6, the random peptide phage display technique appeared to be succesfull for the identification of the epitopes of monoclonal anti-histone autoantibodies. In addition to the epitope of KM-2 described in chapter 3, we also identified the epitope of mAb #34 (**chapter 7**). This resulted into the common motif S/G-A-P-A-S/T-G, which corresponds to amino acid residues 28-33 of the N-terminal region of histone H3. Four different isoforms of histone H3 have been reported: H3.1, H3.2 and H3.3, which have similar sequences, and the

completely different centromeric histone H3 variant, CENPA (231). Recently, the H3.3 variant has been linked to transcriptionally active chromatin regions, while the H3.1 variant is tightly coupled to DNA replication (232, 233). The single amino acid difference between the histone H3 variants H3.1 and H3.3 in the N-terminal tail resides within the epitope we identified for mAb #34, i.e. at position 31 an alanine residue in H3.1 and a serine residue in H3.3. We demonstrated that mAb #34 was specific for H3.1 both in direct and inhibition ELISA using synthetic H3 peptides. Additionally, we tested several modifications, i.e. acetylation, phosphorylation and methylation, which have been described within or close to the identified epitope. We found that acetylation and/or phosphorylation resulted in a decreased binding of mAb #34, while di-methylation had little effect on the binding. However, the effect of the H3.1 modifications on the binding of mAb #34 was much less pronounced when compared to the single amino acid substitution at position 31 in H3.3. Since antibodies specific for histone H3 variants are rare, the specificity of mAb #34 for the H3.1 variant was applied in a study to demonstrate the presence or absence of histone H3 variants in the early mouse zygote (98).

Binding of anti-nucleosome/nucleosome complexes to the C-terminal peptide (83-119) of the SmD1 autoantigen

Anti-nucleosome/nucleosome complexes can bind to both negatively charged (via positively charged histones) and positively charged molecules (via negatively charged DNA). Direct binding of anti-DNA autoantibodies has been described for many proteins, such as alpha-actinin, alpha-enolase, myosin and the glutamate receptor (92, 284-286). However, contamination of the anti-DNA antibody (94) or applied reagents (see also chapter 6) with nucleosomal material easily can lead to misinterpretation of data. In **chapter 8**, we showed that the reactivity with the C-terminal part of the SmD1 autoantigen (SmD1 83-119) is not only due to direct binding of specific anti-SmD1 autoantibodies, but also to indirect binding of anti-DNA antibodies complexed to DNA and/or nucleosomes. DNaseI treatment of both the applied blocking reagent and the patient serum tested resulted in an almost complete abolishment of reactivity with the SmD1(83-119) peptide in several sera from SLE patients, as measured in ELISA. Since the SmD1(83-119) peptide contains about 50% positively charged amino acid residues, anti-DNA/DNA complexes could bind to this peptide via the negatively charged DNA. In fact, we could demonstrate binding of DNA and nucleosomes to the SmD1(83-119) peptide. It has been reported that immunisation with the SmD1(83-119) peptide induces the development of anti-DNA antibodies, which was explained by antigen-spreading (238). In addition, recent results revealed that the SmD1(83-119) peptide can induce

tolerance in SLE mice via the activation of regulatory T-cells (287). Our data suggest that nucleosomes bound to SmD1 may be able to initiate and modulate the autoimmune response to nucleosomes.

Binding of anti-nucleosome/nucleosome complexes to the GBM

Anti-DNA and nucleosome-specific antibodies play a decisive role in the development of lupus nephritis, since complexed to nucleosomes they can bind to the glomerular basement membrane (GBM) and cause local inflamation. In the past, our group has identified and studied this phenomenon extensively (summarized in **chapter 9**). It was found that intrarenal perfusion of autoantibodies complexed to nucleosomes led to glomerular binding and albuminuria, in contrast to perfusion of the same autoantibodies in non-complexed form. In these studies, anti-nucleosome autoantibodies were eluted from glomeruli of diseased lupus mice, and nucleosomes (i.e. histones and DNA) were identified in glomerular depositis in lupus mice. A decrease in staining of heparan sulfate (HS) along the GBM was found in lupus mice with albuminuria. This decreased staining for HS revealed an inverse correlation with immunoglobulin (Ig) deposits in the GBM. This indicated masking of negatively charged HS by Ig complexes. The interaction of anti-nucleosome/nucleosome complexes with heparan sulfate (HS) in the GBM was most likely due to the interaction of cationic residues in the histone tails with anionic residues of HS. Nucleosome/Ig complexes were induced *in vivo* in nude BALB/c mice by intraperitoneal injection of hybridomas producing monoclonal anti-DNA, anti-histone or nucleosome-specific autoantibodies. Immunoglobulin deposits were found much more frequently in mice injected with anti-DNA or nucleosome-specific hybridomas then mice injected with anti-histone hybridomas. This indicated, that especially anti-DNA and nucleosome-specific autoantibodies were nephritogenic. We postulated that especially these autoantibodies by binding can mask the negative charge of the nucleosome, which leads to a more positively charged nucleosome, thus facilitating deposition in the GBM.

Future direction of research

Work from this thesis raised many interesting questions about the role of nucleosomes and anti-nucleosome autoantibodies in the pathogenesis of SLE. Primarily, the identification of **apoptosis-induced histone modifications** as targets for autoantibodies in SLE definitely proves that nucleosomes released from apoptotic cells are able to drive the anti-chromatin immune response as originally was proposed in our model. Future research should be aimed at the identification of additional apoptosis-induced histone modifications that are targeted by SLE autoantibodies. Furthermore, the effect of modified nucleosomes on the maturation of dendritic cells, the processing and presentation by dendritic cells to T cells and the direction of the T and B cell responses to nucleosomes, histones and DNA, should be evaluated. The possible role of these apoptotic modified nucleosomes in binding to the GBM also requires additional research. Since autoantibodies against modified histone peptides were already found in young, pre-diseased lupus mice, a possible diagnostic application of these modified peptides should be further investigated in plasmas from SLE patients. Indeed, autoantibodies that recognize citrullinated proteins, another covalent modification (which has also been described for histones), are specific and predicitive for another autoimmune disease, i.e. rheumatoid arthritis. Importantly, histone peptides containing apoptosis-induced modifications may be used for the induction of tolerance and in this way may offer new therapeutical tools for the treatment of SLE. Promising results have already been obtained with another lupus autoantigen, a phosphorylated U1-70K peptide, which could prevent the development of SLE in MRL/lpr mice (288).

Selection and characterization of mimotopes for nucleosome-specific autoantibodies seemed to be a mission impossible using the current technology. However, the identification of these mimotopes still remains important for diagnostic and therapeutic purposes. Future research should be directed to the development of techniques that circumvent the use of reagents that contain interfering nucleosomal material.

Finally, the **binding of anti-nucleosome/nucleosome complexes** to other proteins probably has an important pathophysiological role in the induction of the autoimmune response in SLE and the induction of tissue damage. Although, many scientists still strongly believe in the direct binding of anti-DNA autoantibodies to various non-nucleosomal proteins, the binding of anti-nucleosome/nucleosome complexes is an attractive alternative for this binding that should be acknowledged. As we have shown in the past and in this thesis the use of anti-chromatin autoantibodies complexed to nucleosomes and/or reagents that

contain nucleosomal material should be circumvented in future experiments studying anti-chromatin autoantibodies. Althoug it was not the intention of our research at the first phase, the identification of mimotopes for nucleosome-specific and anti-DNA autoantibodies as DNA- and nucleosome-binding peptides could lead to a better insight in the pathological effect of these complexes.

Chapter 11.

Samenvatting

Iedere dag is ons lichaam in een strijd gewikkeld met binnendringende microorganismen, waaronder bacteriën, schimmels and virussen. Gelukkig is ons lichaam uitgerust met een verdedigingsmechanisme tegen deze indringers. Dit afweersysteem (of immuunsysteem) is getraind om allerlei structuren te herkennen, echter alleen van indringers, niet van het lichaam zelf. Helaas is dit systeem feilbaar. **Systemische lupus erythematodes (SLE)** is het gevolg van een falen van ons afweersysteem. SLE is een autoimmuunziekte, wat wil zeggen dat het immuunsysteem eigen structuren herkent en hiertegen antistoffen maakt. Aangezien autoantistoffen circuleren in het bloed, kunnen ze leiden tot ontstekingen door het hele lichaam, ondermeer in de huid en de gewrichten, maar ook in organen zoals de nieren, het centrale zenuwstelsel, het hart, de longen en de bloedvaten. Deze ontstekingsreacties veroorzaken de ziekteverschijnselen in SLE, die echter per patient zeer verschillend kunnen zijn. SLE komt vooral voor bij vrouwen (90%) en kan ontstaan op elke leeftijd, maar meestal tussen de 20-40 jaar. De exacte oorzaak van SLE is nog niet bekend. Waarschijnlijk spelen meerdere factoren een rol, waaronder omgevings- (zonlicht), hormonale en genetische factoren en infecties. Aangezien de oorzaak nog niet bekend is kan de ziekte zelf niet genezen worden, de symptomen kunnen wel onderdrukt worden door diverse medicijnen.

Ondanks decennia onderzoek blijft de oorzaak van SLE een puzzel. Tot nu toe zijn er 100 verschillende autoantigenen bekend. **Het nucleosoom** wordt echter gezien als het belangrijkste autoantigeen in deze complexe autoimmuunrespons. Nucleosomen zijn de basisstructuur van chromatine. Chromatin is de structuur waardoor 2 meter DNA in een celkern van ongeveer 20 micrometer kan worden opgevouwen. Een nucleosoom bestaat uit 146 baseparen dubbelstrengs (ds) DNA tweemaal gewikkeld rond een octameer gevormd door paren van de histonen H2A, H2B, H3 en H4. Histonen kunnen op vele manieren gemodificeerd worden, bijvoorbeeld door het aanzetten of verwijderen van een acetyl ((de)acetylering), een fosfaat ((de)fosforylering) of een methylgroep ((de)methylering). Deze modificaties spelen normaal een belangrijke rol in de regulatie van de chromatinestructuur en het activeren en inactiveren van genen.

Wij hebben de afgelopen jaren een hypothese ontwikkeld voor het ontstaan van SLE, waarin het nucleosoom een belangrijke rol speelt (zie hoofdstuk 1 en 2). In het kort gaat dit model als volgt: het lichaam gebruikt **apoptose**, of geprogrammeerde celdood, als mechanisme om cellen die niet meer nodig zijn op te ruimen zonder hun inhoud vrij te laten komen. Dit is zeer belangrijk, aangezien bestanddelen van de cel toxisch kunnen zijn en er tijdens apoptose veranderingen optreden van zowel DNA als allerlei eiwitten. Het immuunsysteem zou deze veranderde, of gemodificeerde, eiwitten kunnen herkennen en antistoffen gaan

maken tegen deze gemodificeerde eiwitten. Normaliter worden apoptotische cellen direct opgeruimd door macrofagen, de vuilnissmannen van ons lichaam. Er zijn de laatste jaren vele aanwijzigingen gevonden dat apoptose en/of de opruiming van apoptotische cellen is verstoord in zowel lupusmuizen als SLE patienten. Wanneer apoptotische cellen niet adequaat opgeruimd worden, vallen ze uit elkaar en komt hun inhoud vrij. Het idee is dat het immuunsysteem antistoffen gaat maken tegen deze gemodificeerde eiwitten, waaronder nucleosomen. In bijna alle SLE patienten worden antistoffen gevonden tegen nucleosomen, bovendien hebben deze patienten ook de vrijgekomen nucleosomen in hun bloed. De anti-nucleosoom antistoffen binden aan nucleosomen en vormen daardoor immuuncomplexen. Deze immuuncomplexen kunnen binden aan allerlei moleculen, waaronder heparansulfaat (dat voor de negatieve lading in het nierfilter zorgt) in de nier. Hierdoor ontstaan er plaatselijk een nierfilterontsteking, een zogenaamde glomerulonefritis. In dit proefschrift hebben we de rol van chromatine en anti-chromatine autoantistoffen in dit model verder geëvalueerd.

Apoptose-geïnduceerde histonmodificaties zijn de drijvende kracht in de ontwikkeling van een anti-chromatine respons in SLE

Nucleosomen kunnen alleen ontstaan tijdens apoptose, waarbij ze gevormd worden door het knippen van chromatin door specifieke enzymen (endonucleases). Apoptose, of geprogrammerde celdood, gaat gepaard met het knippen en modificeren van vele eiwitten. Het is opmerkelijk dat vele van deze gemodificeerde eiwitten in SLE herkend worden door autoantistoffen. Hoewel apoptose-geïnduceerde modificaties op histonen beschreven zijn, is nog nooit aangetoond dat deze gemodificeerde histonen specifiek worden herkend door SLE autoantistoffen. In **hoofdstuk 3** laten we voor het eerst zien dat histonen die tijdens apoptose worden geacetyleerd, specifiek worden herkend door SLE autoantistoffen. We hebben het epitoop van KM-2, een monoklonaal autoantilichaam gericht tegen histonen en afkomstig uit de MRL/lpr muis (een model in de muis voor SLE), gekarakteriseerd door als het ware te 'vissen' in een faagbank, waarin iedere faag een willekeurig kort stukje eiwit (peptide) tot expressie brengt. Wij identificeerden het epitoop op de N-terminale staart van histon H4, maar vonden echter de lysineresiduen op positie 8, 12 en 16 niet terug in het epitoop. De acetylatie van lysines op positie 5, 8, 12 en 16 is beschreven voor histon H4. In de enzyme-linked immunosorbent assay (ELISA) vonden we dat KM-2 de H4 peptides geacetyleerd op positie 8, 12 en/of 16 beter herkent dan het niet geacetyleerde peptide. Vervolgens hebben we de binding van KM-2 bepaald aan histonen afkomstig uit cellen die behandeld zijn met een remmer van histondeacetylases (Trichostatine A, TSA), hetgeen leidt tot een hyperacetylatie

van histonen, en cellen behandelt met camptothecine (CPT, een remmer van DNA topoisomerase I) of een anti-Fas antilichaam die beide leiden tot apoptose van de behandelde cel. Zowel behandeling met trichostatine A, camptothecine als anti-Fas zorgde voor een sterkere binding van KM-2 in de celkern. Dit vonden we ook via analyse in Western blot van celextracten van de behandelde cellen, die een verhoogde reactiviteit van KM-2 met histon H2A en H4 onthulde. Dit betekende dat KM-2 specifiek histon H4 en H2A geacetyleerd tijdens apoptose herkende. Deze resultaten vonden we niet alleen met het monoklonale antilichaam KM-2, maar ook met plasma's van lupusmuizen en SLE patienten. In ELISA bleek dat autoantistoffen in deze plasma's ten opzichte van het ongemodificeerde peptide een verhoogde reactiviteit lieten zien met minimaal een van de geacetyleerde peptides. Bovendien bleek in een analyse met Western blot dat deze autoantistoffen in SLE plasma's een hogere reactiviteit hadden met de histonen H4, H2A, H2B, H3 (en H1) geëxtraheerd uit cellen die behandeld waren met CPT of TSA. Acetylatie en deacetylatie van histonen is een dynamisch proces, waarbij enzymen betrokken zijn die de histonen acetyleren (histonacetyltransferases of HAT's) en enzymen die de histonen deacetyleren (histondeacetylases of HDAC's). De apoptose-geïnduceerde hyperacetylatie van histonen bleek via een verhoogde activiteit van acetyltransferases en een verlaagde activiteit van histondeacetylases te gaan. Uit deze resultaten concludeerden we dat histonen geacetyleerd worden tijdens apoptose en vervolgens het doelwit zijn van autoantistoffen in SLE.

Om het effect van een histon H4 peptide tri-geacetyleerd op de lysineresiduen 8, 12 en 16 (het epitoop van KM-2) op de ontwikkeling van SLE in MRL/lpr muizen te bepalen, hebben we deze muizen ingespoten met een tri-geacetyleerd H4 peptide, een ongemodificeerd H4 peptide of fysiologisch zout (PBS). Behandeling van MRL/lpr muizen met een tri-geacetyleerd H4 peptide leidde tot een duidelijke versnelling van de ontwikkeling van de ziekte, gemeten aan het ontstaan van een eiwitlek in de urine en de ontwikkeling van huidafwijkingen. De behandeling met het ongemodificeerde H4 peptide of PBS versnelde de ontwikkeling van de ziekte niet. Niveaus van anti-chromatine autoantistoffen waren niet verschillend tussen de verschillende behandelingen, maar er was wel sprake van een verhoogde DTH (delayed type hypersensitivity) reactie tegen het tri-geacetyleerde H4 peptide in alle groepen, onafhankelijk van de behandeling. Dit duidt erop dat ook T cellen specifiek het getriacetyleerde H4 peptide kunnen herkennen.

Deze vernieuwende resultaten duiden op een cruciale rol voor apoptose-geïnduceerde histonmodificaties in de pathogenese van SLE. Apoptose-geïnduceerde modificaties van autoantigenen worden normaal niet gezien door het immuunsysteem. Een verstoorde opruiming van apoptotische cellen leidt echter tot het vrijkomen van deze gemodificeerde nucleosomen (zie ook volgende sectie). Dit

kan vervolgens leiden tot een verstoring van de balans tussen tolerantie en autoimmuniteit.

Opruiming van apoptotische cellen: het vrijkomen van gemodificeerde nucleosomen

Normaal gezien worden apoptotische cellen direct opgeruimd door fagocyten (zoals macrofagen), voordat hun inhoud, waaronder (gemodificeerde) nucleosomen en histonen, vrijkomt in het omringende milieu. In toenemende mate wordt de laatste jaren gevonden dat een verstoorde opruiming van apoptotische cellen belangrijk is voor het ontstaan van SLE. Muizen die deficiënt zijn voor moleculen betrokken in het complexe proces van de opruiming van apoptotische cellen, ontwikkelen anti-nucleosoom antistoffen en in sommige gevallen een ontsteking aan de nierfilter (glomerulonefritis). In SLE patiënten zijn er ook aanwijzingen gevonden dat de fagocytose van apoptotische cellen verstoord is. Maar het bewijs voor een verstoorde opruiming van apoptotische cellen in lupus muizen, zoals de MRL/lpr en de (NZWxNZB)F1 modellen, is echter nog onduidelijk. Eerder is al door ons laboratorium gevonden dat de een verstoring van de opruiming van apoptotische cellen niet al aanwezig is in lupusmuizen voor de ontwikkeling van de ziekte. In **hoofdstuk 4** laten we zien dat de opruiming van apoptotische cellen in zieke lupusmuizen verlaagd is. Gebruik makende van een *in vitro* fagocytose-assay hebben we de opname van apoptotische cellen door peritoneale macrofagen gemeten in lupusmuizen voor en na het ontstaan van ziekte. In deze assay maakten we gebruik van serum, apoptotische thymocyten en macrofagen uit dezelfde muis (autoloog). Onder deze condities vertoonden macrofagen van zieke MRL/lpr, (NZWxNZB)F1 en NZB muizen een verlaagde fagocytose. De afgenomen fagocytose was het gevolg van een factor in het serum, aangezien het toevoegen van serum van zieke muizen aan macrofagen en apoptotische cellen van niet-zieke muizen leidde tot een afgenomen fagocytose in de *in vitro* assay. In tegenstelling tot andere studies, vonden we geen constitutief defect in de macrofagen van SLE muizen. We vonden echter wel een verschil wanneer we de fagocytose van apoptotische cellen van verschillende normale muizenstammen vergeleken. Dit zou het gevonden verschil met andere studies misschien kunnen verklaren, omdat de meeste studies als controle stam BALB/c muizen gebruiken die een hogere fagocytotische capaciteit vertonen ten opzichte van andere controle of SLE muizenstammen.

Wij concludeerden dat gedurende de ontwikkeling van SLE de opruiming van apoptotische cellen in lupusmuizen is verminderd door een factor in het serum wat vervolgens kan leiden tot een verergering van de ziekte. Deze serumfactor in

zieke lupusmuizen zou een tekort aan een belangrijke component nodig voor fagocytose of een remmer van de fagocytose kunnen zijn. Er zijn verschillende kanditaten aanwezig in het plasma die betrokken zijn bij de opruiming van apoptotische cellen, waaronder IgM, complement, DNaseI, serum amyloid P (SAP), thrombospondine, C-reactive eiwit, nucleosomen en autoantistoffen.

Anti-nucleosoom en anti-C1q autoantistoffen in patiënten met lupus nefritis

Anti-nucleosoom autoantistoffen worden het meest frequent gevonden in SLE patiënten. De titers van autoantistoffen tegen nucleosomen lijken in SLE patiënten overeen te komen met de activiteit van de ziekte en de aanwezigheid van glomerulonefritis, de meest serieuze aandoening in SLE patiënten. Bovendien kunnen anti-nucleaire autoantistoffen al 10 jaar voor het ontwikkelen van de ziekte worden gevonden in toekomstige SLE patiënten. Autoantistoffen tegen nucleosomen kunnen onderverdeeld worden in drie categoriën: 1) antistoffen die DNA herkennen (anti-DNA); 2) antistoffen die histonen herkennen (anti-histon); en 3) antistoffen die alleen intacte nucleosomen herkennen (nucleosoom-specifieke antistoffen). Binnen de eerste studie naar de behandeling van SLE glomerulonefritis, uitgevoerd door de Nederlandse SLE Werkgroep, hebben we de respons tegen nucleosomen, DNA en histonen bestudeerd in 87 SLE patiënten met een proliferatieve lupusnefritis (**hoofdstuk 5**). Deze patiënten werden behandeld met cyclofosfamide en prednison (CY) of azathioprine, methylprednisolon en prednison (AZA). Deze laatste behandeling werd vergeleken met de meer standaard CY behandeling, omdat cyclofosfamide vaak leidt tot onvruchtbaarheid in deze jonge vrouwelijke patiënten. We maten deze antistoffen bij deze patiënten gedurende het eerste jaar van de behandeling. Wij vonden geen verschillen in het beloop van antistoffen tegen nucleosomen, DNA of histonen tussen de twee behandelingsgroepen. We vonden een reactiviteit boven de achtergrond (+3x SD) tegen nucleosomen in 81% van de patiënten, tegen DNA in 96% van de patiënten, tegen histonen in 23% van de patiënten en tegen complement C1q in 62% van de patiënten. De titer van deze antistoffen ging direct na de start van de behandeling snel omlaag. We vonden een correlatie tussen anti-nucleosoom, anti-DNA en anti-histon reactiviteit, maar niet met anti-C1q reactiviteit. We konden ook een correlatie tussen anti-nucleosoom, anti-DNA en de ziekteactiviteit aantonen. We vonden echter geen correlatie tussen de autoantilichamen en parameters voor de nierfunctie. Wij concluderen dat bijna alle patienten met proliferatieve nefritis positief zijn voor anti-nucleosoom en/of anti-DNA antilichamen, met geen klinische verschillen tussen patiënten met hoge of lage antilichaam titers. Behandeling met cyclophosphamide of azathioprine

induceert een vergelijkbare daling in anti-nucleosoom, anti-DNA en anti-C1q autoantilichamen.

Selectie en identificatie van mimotopen voor nucleosoom-specifieke autoantistoffen: a mission impossible

Hoewel nucleosoom-specifieke autoantistoffen aanwezig zijn voor anti-DNA autoantistoffen in SLE en deze antistoffen ook een belanrijke rol spelen in de ontwikkeling van lupusnefritis, is de specifieke detectie niet eenvoudig. Wanneer nucleosomen worden gebruikt als coating kunnen anti-DNA en anti-histon antistoffen ook binden. Een oplossing hiervoor zou het gebruik zijn van 'mimotopen' voor nucleosoom-specifieke autoantistoffen. Dit zijn peptides die het epitoop van een nucleosoom-specifieke autoantistof nabootsen (mimicken). Bovendien, kunnen mimotopen toegepast worden in de behandeling van SLE, door tolerantie te induceren of door de formatie van anti-nucleosoom/nucleosoom immuuncomplexen te voorkomen. In navolging van mimotopen voor anti-DNA antistoffen, eerder gevonden in andere studies, hebben wij een monoklonale nucleosoom-specifieke antistof (mAb #32) gebruikt om een faagbank te screenen die peptides met een willekeurige sequentie tot expressie brengt (**hoofdstuk 6**). Wij vonden een mimotoop met de aminozuursequentie NRKDWIERTL (MIMO#0 genoemd). Deze sequentie bevat zowel negatieve (D/E) als positieve ladingen (K/R), die respectievelijk DNA (negatief geladen) en histonen (positief geladen) zouden kunnen nabootsen. Tot onze verbazing en onze spijt bleek het geïdentificeerde mimotoop een DNA-bindend motief te zijn in plaats van een epitoop voor mAb #32. Ten eerste, vonden we dat de binding van mAb #32 aan het mimotoop afhankelijk was van een van de reagentia die we gebruikten tijdens de ELISA, namelijk het blokkingsreagens dat gebruikt wordt om aspecifieke binding te voorkomen. Ten tweede, bleek dat het merendeel van onze monoklonale nucleosoom-specifieke, anti-DNA en anti-histon antistoffen kon binden aan het MIMO#0 peptide, weer afhankelijk van de gebruikte blokkingreagentia. Ten derde, na DNase behandeling van de blokkingreagentia was de reactiviteit van alle monoklonale antistoffen met MIMO#0 verdwenen. Ten vierde, konden we de aanwezigheid van DNA en histonen direct aantonen in de verschillende blokkingreagentia. Ten slotte, bleek dat het MIMO#0 peptide in staat was om direct nucleosomaal materiaal (i.e. DNA/histonen) en gelabeld DNA te binden. We moesten concluderen dat het MIMO#0 mimotoop in feite een DNA-bindend peptide is! Belangrijk is dat we dezelfde resultaten vonden voor een DNA mimotoop, eerder geïdentificeerd door Yong-Jiang Sun et al. Ook dit peptide bleek een DNA-bindend peptide te zijn. Dus nucleosomaal materiaal aanwezig in experimentele reagentia,

zoals blokkingreagentia en bacteriële groeimedia, die worden gebruikt tijdens de procedures voorkomen de selectie en een correcte karakterisatie van mimotopen voor nucleosoom-specifieke en anti-DNA autoantistoffen. Daarnaast bleek nucleosomaal materiaal in blokkingsreagentia ook de reactiviteit van autoantistoffen in patiëntensera met mimotopen, maar ook met DNA, histonen en nucleosomen, te verstoren. Tot nu toe blijkt uit onze resultaten dat anti-DNA, anti-histon en anti-nucleosoomreactiviteit het beste kunnen worden uitgevoerd met als blokkingsreagens respectievelijk foetaal calf serum (FCS), DNaseI-behandelde melk en FCS, hoewel het gebruik van deze blokkingsreagentia het probleem niet geheel oplost. Belangrijk is dat de zoektocht naar blokkingsreagentia zonder nucleosomaal materiaal wordt voortgezet. Bovendien is het de vraag of deze techniek gebruikt kan worden voor de identificatie van mimotopen voor anti-chromatine autoantistoffen, ondanks dat er met deze techniek mimotopen voor niet-nucleosomale niet-eiwit mimotopen zijn gevonden.

Anti-histon autoantilichaam is specifiek voor histone variant H3.1

In tegenstelling tot de identificatie van mimotopen voor nucleosoom-specifieke autoantistoffen, beschreven in hoofdstuk 6, bleek het screenen van peptide faagbanken zeer geschikt om epitopen van autoantistoffen gericht tegen histonen te bepalen. In aanvulling op de identificatie van het epitoop voor KM-2 beschreven in hoofdstuk 3, hebben we ook het epitoop gevonden voor de monoklonale autoantistof (mAb) #34 (**hoofdstuk 7**). Dit resulteerde in de identificatie van het S/G-A-P-A-S/T-G motief, dat overeenkomt met de aminozuurresiduen 28-33 van de N-terminale staart van histone H3. Voor histone H3 zijn vier verschillende varianten beschreven: H3.1, H3.2 en H3.3, die een vergelijkbare sequentie hebben en de totaal verschillende centromere histon H3 variant, CENPA. Recentelijk is de H3.3 variant geassocieerd met transcriptioneel actief chromatine, terwijl de H3.1 variant gekoppeld is aan DNA replicatie. Het enige verschil in de terminale staart tussen H3.1 en H3.3 is op positie 31 een alanine voor H3.1 en een serine voor H3.3. We hebben laten zien dat mAb#34 specifiek is voor histonvariant H3.1 in zowel de directe ELISA als in de inhibitie ELISA. Verder hebben we ook een aantal modificaties of combinaties van modificaties, zoals acetylering van lysine 18/23, fosforylering van serine 28 en di-methylering van lysine 27, getest die in of in de buurt van het S-A-P-A-S/T motief gevonden zijn. We vonden dat acetylering en/of fosforylering leidde tot een afname van de binding van mAb #34, terwijl di-methylering nauwelijks effect had. Het effect van deze modificaties op de binding was echter veel minder duidelijk dan de substitie van een aminozuur in H3.3. Aangezien antistoffen die specifiek zijn voor histone H3

varianten zeldzaam zijn, heeft de opmerkelijke specificiteit van dit antilichaam geleid tot het gebruik in een studie naar het voorkomen van histon H3 varianten in vroege zygoten (Van der Heijden et al. Mech Dev, in druk).

Binding van anti-nucleosoom/nucleosoom complexen aan het C-terminale peptide (83-119) van het SmD1 autoantigeen

Anti-nucleosoom/nucleosoomcomplexen kunnen zowel binden aan negatief geladen (via de positief geladen histonen) en positief geladen moleculen (via het negatief geladen DNA). Hoewel directe binding van vooral anti-DNA antilichamen is beschreven voor vele eiwitten, waaronder alpha-actinine, alpha-enolase en de glutamaatreceptor, schuilt er een groot gevaar in het gebruik van gecontamineerde anti-DNA antistoffen en/of gecontamineerde reagentia (zie ook hoofdstuk 6). Dat kan leiden tot misleidende resultaten. In **hoofdstuk 8** laten we zien dat er niet alleen anti-SmD1 autoantistoffen, maar ook anti-DNA autoantistoffen gecomplexeerd aan DNA kunnen binden aan het belangrijkste autoepitoop op het SmD1 antigeen (SmD1(83-119)). Een DNaseI behandeling van zowel de gebruikte blokkingsreagentia als van patiëntensera leidde tot het verdwijnen van bijna de gehele reactiviteit met het SmD1(83-119) peptide bij een deel van de patiëntensera. Bovendien konden we de binding van DNA en nucleosomen aan het SmD1(83-119) peptide aantonen. Aangezien het SmD1 peptide voor 50% bestaat uit positief geladen residuen, kunnen anti-DNA/DNA complexen zeer goed binden via het negatief geladen DNA. Immunisatie van het SmD1 peptide leidt tot de produktie van anti-DNA antistoffen, dat tot nu toe door ‘antigeen-spreiding’ werd verklaard. Bovendien laten recente resultaten zien dat het SmD1(83-119) peptide tolerantie kan induceren in SLE muizen via de activatie van regulatoire T-cellen. Onze data duiden op een mogelijke rol van anti-DNA/DNA complexen gebonden aan SmD1 in de initiatie en modulatie van de autoimmune response tegen nucleosomen.

Binding van anti-nucleosoom/nucleosoomcomplexen aan de GBM

Anti-DNA en nucleosoom-specifieke antistoffen spelen een beslissende rol in de ontwikkeling van lupusnefritis, aangezien deze antistoffen gecomplexeerd met nucleosomen kunnen binden aan het glomerulaire basaalmembraan (GBM) en een locale ontsteking kunnen veroorzaken. In het verleden is dit fenomeen in ons laboratorium voor het eerst gevonden en daarna uitgebreid bestudeerd (samengevat in **hoofdstuk 9**). Intrarenale perfusie van autoantilichamen gericht tegen chromatine en gecomplexeerd met nucleosomen bleek te leiden tot glomerulaire binding en een

eiwitlek, in tegenstelling tot de perfusie van niet-gecomplexeerde anti-chromatine autoantilichamen. In deze studies bleek dat autoantilichamen gericht tegen chromatine geëlueerd konden worden uit glomeruli van zieke SLE muizen, en dat nucleosomen (i.e. histonen en DNA) werden gevonden in glomerulaire deposities in SLE muizen. Een afname van heparansulfaat werd gevonden in de GBM van muizen met een eiwitlek, Deze proteinurie vertoonde een negatieve correlatie met immunoglobulline (Ig) deposities in de GBM. Dit duidt waarschijnlijk op een maskering van het negatief geladen heparansulfaat (HS) in de GBM door de interactie van cationische residuen in de histonen en anionische residuen in HS, leidend tot een verstoring van de filterfunctie van de glomerulus en het ontstaan van een eiwitlek. Nucleosoom/Ig complexen werden *in vivo* geïnduceerd door 'nude' BALB/c muizen intraperitoneaal in te spuiten met hybridoma die monoklonale anti-DNA, anti-histon en nucleosoom-specifieke autoantistoffen produceerden. Immuunglobuline deposities werden vaker gevonden in de muizen geïnjecteerd met hybridoma die antilichamen tegen DNA of nucleosomen produceerde, vergeleken met muizen geïnjecteerd met hybridoma die antilichamen tegen histonen produceerde. Hieruit bleek dat vooral anti-DNA en nucleosoom-specifieke autoantistoffen schadelijk voor de nier waren. Wij postuleerden dat de binding van deze antilichamen de negatieve lading van het nucleosoom maskeren, wat leidt tot een meer positief geladen nucleosoom, dat vervolgens de depositie in de GBM mogelijk maakt.

Toekomstig onderzoek

De studies beschreven in dit proefschrift roepen een aantal interessante vragen op over de rol van chromatine en anti-chromatine autoantilichamen in de pathogenese van SLE. De bevinding dat **apoptose-geïnduceerde histonmodificaties** het doelwit zijn van autoantistoffen in SLE bewijst dat nucleosomen die vrijgekomen zijn uit apoptotische cellen een belangrijke prikkel zijn voor de immuunrespons tegen chromatine, zoals oorspronkelijk was voorgesteld in ons model voor de initiatie van SLE en SLE nefritis. Toekomstig onderzoek zou moeten leiden tot de indentificatie van meer apoptose-geïnduceerde modificaties op histonen die herkend worden door SLE autoantistoffen. Het effect van apoptose-gemodificeerde nucleosomen op de maturatie van dendritische cellen, de processing en presentatie door dendritische cellen aan T-cellen en de regulatie van de respons van T en B cellen tegen nucleosomen, zou onderzocht moeten worden. De mogelijke rol van gemodificeerde nucleosomen in de binding aan de GBM vraagt ook om aanvullend onderzoek. Aangezien antistoffen tegen gemodificeerde histonpeptides gevonden zijn in jonge SLE muizen zou een mogelijke diagnostische toepassing in SLE patienten onderzocht moeten worden. In reumatoïde artritis zijn er al autoantilichamen tegen gemodificeerde antigenen gevonden, die specifiek en voorspellende zijn voor deze ziekte. Belangrijk is dat histonpeptides die apoptose-geïnduceerde modificaties bevatten, mogelijk gebruikt kunnen worden om tolerantie op te wekken. Dit zou kunnen leiden tot de ontwikkeling van nieuwe middelen voor de behandeling van SLE. Veel belovende resultaten zijn er al verkregen met een gefosforyleerde U1-70K peptide (een ander autoantigeen in SLE), dat de ontwikkeling van SLE in MRL/lpr muizen kon remmen.

Selectie en karakterisering van mimotopen voor nucleosoom-specifieke autoantistoffen bleek een onmogelijke opgave. De identificatie van deze mimotopen blijft echter belangrijk voor diagnostische en therapeutische doeleinden. Toekomstig onderzoek zou gericht moeten zijn op de ontwikkeling van reagentia die niet gecontimeerd zijn met nucleosomaal materiaal.

Ten slotte heeft de **binding van anti-nucleosoom /nucleosoomcomplexen aan andere eiwitten** mogelijk een pathofysiologische rol in de inductie van de autoimmuunrespons in SLE, vooral met betrekking tot ‘epitoop-spreiding’ en in het ontstaan van weefselschade. Hoewel de meeste wetenschappers nog steeds geloven in de directe binding van anti-DNA autoantistoffen, is de binding van anti-nucleosoom/nucleosoom complexen een attractief alternatief. Zoals we in het verleden en in deze thesis hebben laten zien zou

het gebruik van antistoffen gericht tegen chromatine die gecontamineerd zijn met nucleosomaal material en/of het gebruik van reagentia die nucleosomaal materiaal bevatten, moeten worden voorkomen. Hoewel niet de intentie van dit onderzoek in eerste instantie, zou de identificatie van mimotopen voor nucleosoom-specifieke en anti-DNA autoantilichamen als DNA-bindende peptides kunnen leiden tot een beter inzicht in het pathogene effecten van chromatine/anti-chromatinecomplexen.

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Abbreviations

bp	base pairs
BSA	bovine serum albumin
CPT	camptothecin
DNaseI	deoxyribonuclease I
dsDNA	double stranded deoxyribonucleic acid
DTH	delayed hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GBM	glomerular basement membrane
HAT	histone acetyltransferase
HDAC	histone deacetylase
HS	heparan sulfate
Ig	immune globullin
kDa	kilo Dalton
MAP	multi antigen peptide
Mϕ	macrophage
PBS	phosphate buffered saline
PI	propidium iodide
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
TSA	Trichostatin A

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('Cross-reactivity revisited…'), en uiteindelijk moeten we iedereen kunnen overtuigen.

Cor, ik ken je nu alweer zo'n 8 jaar. Als HLO-broekie kwam ik tijdens mijn stage als eerste bij jouw terecht. Ik kan mij de eerste dag nog herinneren. Tijdens de kennismaking met de mensen van CHL was de reactie meestal: "Moet jij met Cor gaan werken? Nou veel sterkte dan…". Maar dat viel ontzettend mee. Zowel tijdens mijn stage als later tijdens mijn promotie onderzoek was je een belangrijke steun. Zeker ook buiten het werk... Wij hebben nu alweer vele pool-avonden erop zitten en in al die jaren is er niet veel veranderd, de ballen vliegen nog steeds de zaal rond. Tijdens één van die pool-avonden kwam jij ook met het idee of de AIO-baan bij Nierziekten niet iets voor mij was… Jij was ook de grote organisator achter de jaarlijkse lab-BBQ en de sinterklaas/kerstmis-cadeau-doorgeef-spelavond op jouw 'landgoed' in Groesbeek. Meestal mocht ik dan overnachten in 1 van de slaapkamers van jouw ' villa' , en ondertussen heb ik al een eigen tandborstel verworven die nog volgens mij steeds in de la van het nachtkastje ligt. Bedankt voor alle gezelligheid!

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Jürgen

Curriculum Vitae

Jürgen Wilhelmus Carolus Dieker werd op 6 januari 1976 geboren te Etten (Gld). In 1988 begon hij zijn opleiding voor het VWO aan het Rhedens Lyceum in Roozendaal, waar hij zijn diploma behaalde in 1994. Vervolgens vervolgde hij zijn studie tot biochemisch research analist aan het Hoger Laboratorium Onderwijs (HLO) van de Hogeschool Arnhem en Nijmegen, waarbij hij als afstudeerrichting Biochemie koos. Gedurende deze opleiding liep hij stage in het Laboratorium Nierziekten van het Radboud Universiteit Nijmegen Medisch Centrum. Hij behaalde zijn diploma aan het HLO in 1998 en ging vervolgens aan het werk als research analist bij de afdeling Pathologie. In mei 2000 keerde hij terug naar het Laboratorium Nierziekten en begon gesubsidieerd door de Nierstichting aan zijn promotie onderzoek, beschreven in dit proefschrift. Sinds 1 april 2005 is hij werkzaam als Postdoc op de afdeling Immunologie et Chimie Thérapeutique van het Institute de Biologie Moléculaire et Cellulaire (IBMC) in Straatsburg (Frankrijk). De eerste drie maanden gesubsidieerd door het FEBS (Federation of European Biochemical Societies) en vervolgens door het CNRS (Centre Nationale de Recherche Scientifique).

Jürgen Wilhelmus Carolus Dieker was born on January 6th in Etten (Gelderland, The Netherlands). In 1988, he started his education for the VWO at the Rheden Lyceum in Roozendaal, where he received his diploma in 1994. He continued his study for biochemical research technician at the HLO of the Hogeschool Arnhem en Nijmegen (HAN), and he choose Biochemistry as main subject. During his study he performed a traineeship at the Nephrology research Laboratory at the Radboud University Nijmegen Medical Centre. He graduated from the HLO in 1998, and started working as a research technician at the Pathology Department. In May 2000, he returned to the Nephrology Research Laboratory to start his Ph.D. research subsidized by the Dutch Kidney Foundation, as described in this thesis. Since April 1st 2005, he has been working as a Postdoc at departement of 'Immunologie et Chimie Thérapeutique' at the 'Institute de Biologie Moléculaire et Cellulaire' (IBMC) in Strasbourg (France). First on a project granted by the FEBS (Federation of European Biochemical Societies) and next on a project granted by the CNRS (Centre Nationale de Recherche Scientifique).

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